TOXIC EFFECTS OF AN ANIONIC DETERGENT ON THE GILLS OF TROUT

by

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Submitted for the degree of Doctor of Philosophy,

University of Aston in Birmingham.

September 1974

SUMMARY

The literature on the toxicity of synthetic detergents to fish and aquatic invertebrates is reviewed.

The pathological effects of an anionic detergent, sodium lauryl sulphate, on the gills of rainbow trout have been studied by light and electron microscopy. At lethal concentrations up to about 120 mg/l, epithelial cell death is associated with lysosome formation. Acute inflammation of the gill tissue, extensive detachment of the epithelium and collapse of the pillar cell system occur. At concentrations above about 120 mg/l, very rapid lysis of cells results in the complete disruption of cellular and tissue structure. Changes in the gross structure of the gills are explainable in terms of the rate and nature of toxic action at the cellular level. Review of the biomedical literature suggests that the observed effects of SLS on gill cells correspond to the two mechanisms by which detergents cause death in isolated cells. These are autolysis, i.e. lysis by the action of the cells' own enzymes, induced by an initial lesion in the cell membrane whose precise nature is not known; and rapid lysis by the direct action of the detergent on the cell constituents. The occurrence of these two modes of lethal toxic action is also indicated by probit analysis of toxicity data. At sub-lethal concentrations of SLS, below about 30 mg/l depending on the species and/or age of the fish, the epithelium of the secondary lamellae becomes swollen and abnormally detached, its total thickness increasing to about three times its normal value. There is a redistribution of chloride cells and sub-acute inflammation of the gill tissue occurs. These anatomical changes probably impair respiratory exchange to a significant extent.

The findings are discussed in relation to areas of general interest in fish toxicology.

ACKNOWLEDGMENTS

These investigations were carried out during the tenure of a University of Aston Postgraduate Research Scholarship. I wish also to record my gratitude to the following individuals:

Dr J. F. Skidmore, supervisor, for his advice and criticism throughout the project.

Mr C. U. M. Smith for guidance in the principles and techniques of electron microscopy.

Prof. T. Mulvey of the Department of Physics, who allowed me the use of the electron microscope in his charge for a period of several months while the EM6B was out of commission.

Mr S. B. Southwick, who on his appointment in December 1972, rapidly restored the EM6B to a serviceable condition after months of neglect, and thereafter maintained it and its ancillary equipment in excellent order on a shoestring budget. I am also grateful for his readiness to assist in technical matters, and his ingenuity in maintaining supplies of essential materials.

Mr J. A. L. Fraser for the loan of photographic equipment.

Miss P. F. Barry, who typed the final manuscript.

My wife, Kate, for countless hours of assistance in preparing this thesis, and for her invaluable support and encouragement.

Contents

INTRODUCTION

With very few exceptions, well-developed gills are characteristic of all the 20,000 or more known fish species. In teleosts, gill functions are not confined to respiration, but also include osmotic and ionic regulation and excretion of ammonia. The anatomy of teleost gills has therefore been extensively studied as a necessary adjunct to the many physiological investigations; and as a result their morphology and fine structure are well known, both in the typical form and in a number of striking adaptations.

Several aspects of the typical gill structure are of importance here. Firstly, the great area of the gills, often amounting to several times the body area (Gray, 1954); secondly, the slightness of the barrier between the internal and external environment, in the Salmonidae perhaps as little as one micron; and thirdly, the large quantity of water which is passed over the gills. These features are all necessitated by the scant amount of oxygen to be had from a volume of water, and their most important implication in the present context is that the gills are morphologically and functionally by far the most important source of contact between the fish and its environment.

It is for these reasons that study of the effects of pollutants on gills is potentially of value. Discerning studies may be able to detect quite small deviations from the normal structure, particularly in relation to the action of pollutants at the cellular level. Although the phenomenon of pollutant-induced gill damage is well known, it has not hitherto been studied in depth. The studies described here are largely based on light and electron microscopical investigations of the response of trout gills to a detergent. However, little research comparable to that described here has been carried out in relation to any pollutant, so it is hoped that these studies will be of value in fish toxicology generally, rather than exclusively in the context of any one group of poisons.

A detergent was chosen as experimental toxicant for several reasons. The only toxicants whose effects on fish gills have been studied electron microscopically are the heavy metals zinc and copper; detergents are sufficiently far removed chemically for a comparison of their effects to be of interest. Detergents are readily soluble in water. There is a fairly extensive literature on the toxicity of detergents to fish, and the occurrence of gill damage is well documented. Indeed, the interactions between detergents and living organisms has been an active area of research for the last forty years. Sodium lauryl sulphate has been employed because it is typical of the anionic detergents, the most widely-used and best-studied group; because it is one of the few such substances readily available in a highly-purified state; and because preliminary experiments showed its toxicity to range over usefully wide limits of concentration and survival time.

The thesis is divided into six chapters. Chapter 1 is a review of the literature to date on all aspects of the toxicity of detergents to fish. This is intended to relate the content of the subsequent chapters to the context of preceding research, and to demonstrate that although detergents are now no longer regarded as a major water pollution problem, the study of their effects can remain of interest and value in fish toxicology. Chapter 2 describes the gill damage in trout exposed to an acutely toxic concentration of sodium lauryl sulphate (SLS). In the light of these observations, previous studies of pollutant-caused gill damage are reviewed and the nature and mechanism of the gill reaction is discussed with special reference to generalised and specific features of the response. Chapters 3 and 4 describe studies of gill damage in relation to the survival time of fish in several acutely-lethal concentrations of detergent. Chapter 5 reports the effects on gill morphology of sublethal concentrations of detergent and partially-degraded detergent. This is followed by a general discussion

in Chapter 6 in which the results of these investigations are related to matters of current general interest in fish toxicology.

Chapter 1 of this thesis form the substance of a paper already published in the Journal of Fish Biology (Abel, 1974). Chapter 2 is based on a paper recently accepted for publication in Water Research. Throughout this thesis all figures and tables are inserted at the end of the appropriate chapter.

CHAPTER 1. Review of the literature on the toxicity of synthetic detergents to fish and aquatic invertebrates.

1.1 INTRODUCTION

The effects of pollution by synthetic detergents on aquatic life have been reviewed as part of wider surveys by Patton (1963) and Prat & Giraud (1964); and more exclusively by Marchetti (1965a). Detergents became widespread pollutants in the 1950's, from which time interest arose in their toxicity. The general introduction in 1965 of rapidly biodegradable detergents promoted research on new compounds. This review surveys the major literature of the last fifteen years on the toxicity of detergents to freshwater and marine fish. Some literature on invertebrates is also included, since their consideration is relevant in relation to water quality criteria. The major aspects discussed are the determination of toxic concentrations and the factors which influence their toxicity; the definition, detection and description of lethal and sublethal toxic effects; and the mode of action of detergents.

Synthetic detergents are a diverse group of compounds, and part of a larger group known as surface-active agents or surfactants. Their detailed chemistry is beyond the scope of this discussion, and readers are referred to the accounts of Swisher (1970) and Silsby (1968) for recent reviews of their nomenclature and chemical properties. However, certain salient features will be described briefly. The otherwise diverse molecular forms of detergents have in common a hydrophilic-hydrophobic polarity whence stem three important general properties: the tendency to concentrate at surfaces, the reduction of surface tension in solution, and the formation of aggregates of ions, micelles, when present in solution above a certain critical concentration. These properties are related aspects of the phenomenon of surface activity.

Anionic detergents are the most-studied group, since they are the most widely used and represent the major source of detergent pollution.

The most common anionic detergents are the alkyl aryl sulphonates such as alkylbenzene sulphonate (ABS), and alkyl sulphates. An ABS with an unbranched hydrocarbon chain is usually referred to as linear alkylate sulphonate or LAS. Alkyl sulphates and LAS detergents figure prominently among the soft (rapidly biodegradable) detergents. ABS derived from tetrapropylene and having a branched hydrocarbon chain is more resistant to biodegradation, and is thus more persistent in the environment. For this reason, LAS is by convention distinguished from ABS, although LAS is an alkylbenzene sulphonate. Non-ionic detergents, although used in significant quantities, have attracted rather less attention than anionics. Cationic detergents are mainly used as medical and laboratory disinfectant agents, and for other specialised purposes. Since they are scarcely used and cannot co-exist in solution with the more prevalent anionics (Haney et al., 1954) they have so far had little widespread environmental significance.

Although the majority of detergents in use since 1965 have been of the soft type, research on hard detergents remains of value except in relation to water quality criteria. When considering the response of animals to toxic agents, the mode of action of toxic agents and the toxicity of substances in relation to chemical structure and physical properties, the data accumulated on hard detergents remain perfectly valid and useful. A purely pragmatic approach to fish toxicology too frequently appeals to those concerned with water quality, impeding the study of fundamental biological phenomena of great potential importance in the longer term. This review, while considering water quality criteria, attempts also to discuss the intoxication of fish by detergents as a biological phenomenon.

1.2 MEASUREMENT OF TOXIC CONCENTRATIONS

A. Methods

Sprague (1969, 1970, 1971) has recently reviewed the methods and practice of fish toxicology. Most of the studies reviewed here have adopted the rigorous approach Sprague outlines. Many earlier studies, which can be traced through the reviews referred to above, and some recent unrefined ones are difficult to compare meaningfully with more recent work and are not discussed.

Sprague's recommendations include not only the mechanics of toxicity testing, but also the underlying approach to it. In particular, he urges the use of the incipient LC50 (synonymous with lethal threshold concentration and incipient lethal level) as the criterion of acute toxicity, or the 96h LC50 as an acceptable substitute in most cases. Table 1.1 shows representative toxicity values for various fish species and different detergents, and includes only those results obtained in accordance with Sprague's (1969) recommendations. The list includes most of the results reported which are considered reliable.

Detergents differ from most toxicants in that one name may embrace a large number of different compounds. Swisher (1963) points out that ABS has 80,000 isomers in the range C_{10} - C_{15}, and that C_{12} ABS alone has 3,057 isomers. Commercial ABS contains between 75 and 100 major components detectable by gas chromatography. The precise nature of a sample may not be known even to its manufacturer; unbranched ABS (LAS) is, however, easier to characterise, C_{12} LAS having as few as five isomers. The multiple character of detergents explains much of the variation in reported results: the influence of molecular configuration on toxicity is discussed later. Detergent toxicants should be described as completely as possible, including their source and purity and the length and nature of the hydrocarbon chain. Frequently detergents are available only in impure commercial formulations: Herbert et al. (1957) and Henderson et al. (1959) showed that common detergent additives and

contaminants contribute little to the toxicity of these. For research purposes, however, the use of pure surfactant where possible seems desirable, not least because results are more easily comparable.

Detergents concentrate at surfaces, and under test conditions will concentrate at the sides of the vessel and at the air-water interface. The significance of the non-uniform distribution of toxicant in solution has not been investigated, but excess foaming should be avoided to minimise the area of the air-water interface. Detergent molecules may also be expected to concentrate on the surfaces of the fish exposed to water. This is of unknown significance in the toxic action of detergents, but its possible importance is obvious from the consideration that any toxic substance must make contact with the fish in order to exert its effect. The work of Lloyd & Herbert (1960) on ammonia toxicity indicated the importance of considering toxicant concentration at the gill surface rather than in the water generally.

Biological and chemical degradation of the detergent is likely in all but the shortest tests, especially when using soft detergents. Many authors have reported loss of toxicant during tests: it is therefore important that results are based on measured rather than calculated concentrations, and that concentrations are maintained within reasonable limits. A practical difficulty of significance both in toxicity testing and in setting water quality criteria is that it is not known whether quantities of detergent producing equal responses in chemical tests are equally toxic if they are in different states of degradation. What evidence exists (Herbert et al., 1957; Swisher et al., 1964) indicates that partially degraded detergent is less toxic than raw detergent. This accords with the relationship between toxicity and molecular structure discussed later.

There does not appear to be any substantial difference between the results obtained from static and continuous flow bioassays, provided the

tests are properly conducted, (Cairns & Scheier, 1962; Hokanson & Smith, 1971). Sprague (1969) has suggested that static tests may tend to show thresholds in shorter times, but there is insufficient evidence to test this hypothesis fully.

B. The survival time-concentration relationship.

There has been no satisfactory study of the relationship between survival time and concentration for fish exposed to detergents. Published curves are indicative of the general range over which detergents are toxic, but cannot be regarded as valid descriptions of the time-concentrations relationship because they are based on an insufficient number of points, because those points lack confidence limits, or because confidence limits when plotted are ignored when fitting the toxicity curve. Time-concentration relationships have been published in the form of hyperbolic curves (Hokanson & Smith, 1971; Swedmark et al., 1971), straight lines with thresholds (Wildish, 1972) and straight lines without thresholds (Herbert et al., 1957); but with the possible exception of the latter case, all appear largely to lack justification. A further limitation of all published curves except those of Wildish (1972) is the lack of information concerning survival time at high concentrations, no doubt owing to an understandable preoccupation with water quality criteria but unfortunate from a more fundamental point of view.

What does emerge from these curves is the indication that detergents may continue to act lethally over long periods of time. No lethal threshold was evident after 12 weeks for Salmo gairdneri (Richardson) exposed to ABS (Herbert et al., 1957). Lethal action of LAS on Gadus morrhua (Linn.) and Pleuronectes flesus (Linn.) did not cease in 8 days (Swedmark et al., 1971); of ABS on the same species in 20 days. Apparent lethal thresholds occurring within 24 or even 48 hours may not be the ultimate lethal thresholds: Sprague's (1971) strictures regarding the

necessary duration of toxicity tests appear amply justified in the case of detergents.

Sufficiently high concentrations to allow the determination of a minimum time for lethal action have rarely been tested. Many of the curves shown by Swedmark et al. (1971) for marine fish exposed to five detergents indicate an apparent threshold of this type occurring between 0.5 and 1 hour. These are probably not true thresholds, since the highest concentrations tested ranged from 32 - 100 mg/l and there are insufficient points to justify the construction of an asymptote on the concentration axis. A more appropriate estimate of minimum times for lethal action is probably in the order of ten minutes or less. Wildish (1972) obtained a similar figure for Salmo salar (Linn.) exposed to 100 mg/l of non-ionic polyoxyethylene (4) lauryl ether; a value in this range is also found when trout are exposed to high concentrations (above 300 mg/l) of anionic sodium lauryl sulphate (see Chapters 3 and 4).

C. Factors affecting acute toxicity

The LC50 values in Table 1.1 vary over two orders of magnitude. (A report by Brown et al. (1968) of the most toxic detergent so far studied has been omitted from Table 1.1 because the detergent, described as ABS-type, was otherwise unspecified. Its 5-day LC50 to S. gairdneri was 0.4 mg/l). Different authors frequently report widely different toxic concentrations for the same species. The biotic and abiotic factors which affect toxicity have not been standardised, so that comparison of results between different investigators is virtually impossible. Proprietary detergents bearing the same name may behave quite differently. However, various workers have investigated a number of factors affecting toxicity, and their results will be considered in some detail.

1. Detergent type

Table 1.1 shows that anionic and non-ionic detergents are toxic over similar ranges of concentration. There is no evidence to support the occasionally-made generalisation that non-ionic detergents are less toxic to fish than anionics.

The configuration of the detergent molecule greatly influences toxicity. This effect is best known with regard to anionic detergents. It is well established that linear alkylbenzene sulphonates are more toxic than the hard alkylbenzene sulphonates, though the more rapid biodegradation of LAS may tend to compensate in the field for its higher toxicity. Hirsch (1963) appears to have been the first to show LAS to be more toxic than ABS, recording 48h LC50 values for golden orfe (Idus idus Linn.) of 4 and 13 mg/l respectively. His results have since been confirmed for a wide range of species (Table 1.1), for example by Pickering (1966), Thatcher & Santner (1967) and Swedmark et. al. (1971).

Chain length is an important factor in detergent toxicity. Hirsch (1963) found the toxicity of LAS to Idus idus increased with chain length from C_8 to C_{15}, decreasing at C_{16} (Fig. 1.1). Swisher et al.(1964) showed C_{14} LAS to be more toxic than C_{12} LAS to Lepomis macrochirus (Rafinesque) (Table 1.1), while a degradation product was much less toxic than either. Wildish (1972) found non-ionic polyoxyethylene ether with four units of ethylene oxide per molecule more toxic than one with 23 units to Salmo salar (Table 1.1)

2. Environmental factors

A large literature exists on the effects of various environmental factors on the toxicity of poisons to fish. The inferences drawn from studies of this type usually centre on water quality standards in respect of different environments, and the possibility that factors affecting toxicity may provide clues to the mechanism of action of the poison is too often overlooked. Unfortunately the fundamental studies

required by this approach are usually lacking: for example, an environmental factor may affect the test organism, the chemical state of the toxicant, or both. Detergents are no exception to the general rule in that factors modifying their toxicity have been investigated, but with little resultant enlightenment from the toxicological viewpoint.

Water hardness is an important factor in the toxicity of many poisons, and the toxicity of detergents has been variously reported to increase, decrease or be unaffected by increasing water hardness. Henderson et al. (1959) found ABS more toxic to Pimephales promelas (Rafinesque) in hard water than in soft, but a sodium alkyl sulphate was more toxic in soft water. The toxicity of a non-ionic polyoxyethylene ester was unaffected by water hardness. Cairns & Scheier (1962) found the toxicity of ABS not significantly affected by water hardness. In contrast, Hokanson & Smith (1971) found water hardness "the most significant environmental variable" in the toxicity of LAS to Lepomis macrochirus, mean incipient LC50's being given as 4.25 mg/l and 2.85 mg/l in waters of hardness 15 mg/l and 290 mg/l $CaCO_3$ respectively. Tovell et al. (1974) found that the uptake of radio-actively labelled sodium lauryl sulphate (SLS) by rainbow trout and goldfish was greater in hard water than in soft water. Toxicity was measured by a rather unsatisfactory method, although the results did correspond to the pattern of detergent uptake, SLS being more toxic in hard than in soft water. The formation of a calcium-SLS complex more easily absorbed by the fish was suggested as a possible mechanism of increased toxicity: this mechanism is discussed more fully below.

Unlike heavy metals, which are uniformly more toxic in soft than in hard water, there is no reason to suppose that detergents will behave uniformly with respect to water hardness. The majority of reports state that anionic detergents are more toxic in hard than in soft water, though there is considerable disagreement on this point. If increased toxicity, where it occurs, is indeed due to the formation of a complex with

calcium ions, the source of the discrepancies may lie in the detailed chemistry of the various test solutions, for example in the precise states of equilibrium as between the various ions and complexes present.

Information on the influence of temperature on detergent toxicity is scarce. Hokanson & Smith (1971) found temperature exerted no significant effect on the threshold toxic concentration of LAS to Lepomis macrochirus, (2.35 mg/l at 15°C, 2.23 mg/l at 25°C), though the time of lethal threshold was reduced from 51h at 15°C to 24h at 25°C. Swedmark et al. (1971) found that toxicity of both LAS and nonylphenol ethoxylate to Gadus morrhua, Pleuronectes flesus and some marine invertebrates was greater at 15-17°C than at 6-8°C, in terms of 96h LC50. However, these authors also recorded that lethal action of the detergents on fish had not ceased in 96 hours, (see above), and the effect of the increased temperature may simply have been to increase the speed of action of the poison, as was the case in Hokanson & Smith's (1971) experiments. Sprague (1970) has indicated other cases where insufficient testing has produced misleading results: it is particularly important in investigating temperature effects to determine lethal threshold concentrations.

Dissolved oxygen concentration markedly affects the toxicity of many poisons. For detergents, Herbert et al. (1957) reported that median periods of survival of Salmo gairdneri exposed to ABS with 4 mg/l dissolved oxygen present were about half as long as when 8 mg/l dissolved oxygen were present. Hokanson & Smith (1971) measured the lethal threshold concentration of LAS to Lepomis macrochirus at several levels of dissolved oxygen. They found the lethal threshold concentration dropped with dissolved oxygen concentration, at 2 mg/l dissolved oxygen being only 10-20% of the lethal threshold concentration at oxygen saturation. However, it is likely that at the test temperature of 25°C, the very low dissolved oxygen concentration itself exerts a toxic, or at least a limiting, effect. At more moderately reduced dissolved oxygen

concentrations, the toxicity of LAS to L. macrochirus is affected similarly to that of the poisons tested by Lloyd (1961) against Salmo gairdneri, although the regular relation found by Lloyd is not discernible.

Salinity is an important environmental factor and one which has not been sufficiently investigated in relation to the role of osmoregulatory stress in toxicant action. The toxicity of poisons appears to vary in different ways with salinity (Brown et al., 1967), but the only investigation of detergents is that of Eisler (1965), who found that mortality among Fundulus heteroclitus (Linn.) and Anguilla rostrata (le Sueur) exposed to a 96h LC50 of ABS was greatest at high and low salinities.

3. Biotic factors

Comparisons between species must always be made with caution (Sprague, 1970). Many authors have reported toxicity levels for more than one species (Table 1.1). Eisler (1965), Thatcher (1966) and Thatcher & Santner (1967) compared the greatest number of species, and found a four-fold difference in sensitivity to detergents between the most and least resistant species. This range corresponds well with that reported for most other poisons (Sprague, 1970).

Fish appear to be able to acclimate to detergents. Lemke & Mount (1963) exposed Lepomis macrochirus to 3 mg/l ABS for 30 days, and found their 48h LC50 63% higher than that of control fish. Fish acclimated to lower concentrations yielded lower LC50 values. Hokanson & Smith (1971) found for the same species acclimated to 0.5 mg/l LAS an increase in lethal threshold concentration and a decrease in the time of lethal threshold. Lethal threshold concentrations of acclimated fish were 50% higher at air saturation and 400% higher at 2 mg/l dissolved oxygen. Acclimation to LAS tends to cancel out the effects of low dissolved oxygen concentration, and under continuous exposure LAS toxicity to L. macrochirus was not influenced by oxygen levels.

Several investigations have attempted to identify the most sensitive stage in the life cycle. Pickering & Thatcher (1970) found the most critical stage in the life cycle of Pimephales promelas to be the newly-hatched fry, 7-14 days old. Swedmark et al. (1971) reported that the eggs and larvae of plaice and cod were more sensitive to detergents than the adults. Hokanson & Smith (1971) found that Lepomis macrochirus was most sensitive to LAS at the stage of yolk sac absorption.

Pickering (1966) measured the 9-day LC50's of LAS and ABS to fry and juvenile fish, and found 1-day old fish to be most sensitive. Adult

ERRATUM

The first sentence of the second paragraph on page 14 should read :

" Pickering (1966) measured the 9-day LC50's of LAS and ABS to Pimephales promelas eggs. He also measured the toxicity of ABS to fry and juvenile fish, and found 1-day old fish to be most sensitive."

be a link between susceptibility to poisons and the metabolic changes occuring during development. Thus for zinc (Skidmore, 1967), resistance of Brachydanio rerio (zebra fish) of different ages is inversely proportional to the routine rate of oxygen uptake. Water metabolism has been suggested by Marchetti (1965b) as a possible basis for the variation with age in resistance to detergents. The developing fish absorbs water rapidly from the environment as material is transferred from the yolk to the more dilute embryo. The decline in resistance to nonylphenol

Several investigations have attempted to identify the most sensitive stage in the life cycle. Pickering & Thatcher (1970) found the most critical stage in the life cycle of Pimephales promelas to be the newly-hatched fry, 7-14 days old. Swedmark et al. (1971) reported that the eggs and larvae of plaice and cod were more sensitive to detergents than the adults. Hokanson & Smith (1971) found that Lepomis macrochirus was most sensitive to LAS at the stage of yolk sac absorption.

Pickering (1966) measured the 9-day LC50's of LAS and ABS to fry and juvenile fish, and found 1-day old fish to be most sensitive. Adult P. promelas were reported less sensitive to ABS than their eggs. Hokanson & Smith (1971) tested the toxicity of LAS to Lepomis macrochirus sperm by estimating the effect of the detergent on the mean duration of active swimming and total mobility. Fifty per cent reduction of active swimming time was produced by a concentration of 5.7 mg/l, the corresponding figure for total motility being 5.4 mg/l. Tests of fertilisation in the presence of LAS showed that fertility was not limited until nearly all sperm had been rendered non-motile, indicating that fertilisation was not the most critical stage in the life cycle. Marchetti (1965b) found that the 6h LC50 for Salmo gairdneri exposed to nonylphenol ethoxylate had a minimum value, again at the stage of yolk sac absorption.

The evidence favours the view that the most sensitive stage in the life history occurs early in the post-hatching phase. Other poisons, such as zinc (Skidmore, 1965) probably behave similarly, and there may be a link between susceptibility to poisons and the metabolic changes occuring during development. Thus for zinc (Skidmore, 1967), resistance of Brachydanio rerio (zebra fish) of different ages is inversely proportional to the routine rate of oxygen uptake. Water metabolism has been suggested by Marchetti (1965b) as a possible basis for the variation with age in resistance to detergents. The developing fish absorbs water rapidly from the environment as material is transferred from the yolk to the more dilute embryo. The decline in resistance to nonylphenol

ethoxylate was found to coincide with the period of increased water permeability.

4. Detergents and other pollutants

Cairns & Scheier (1964) found prior exposure to ABS did not affect the toxicity of zinc or alter tolerance to high temperature in Lepomis gibbosus. Brown et al. (1968) found that chronic exposure of Salmo gairdneri to zinc did not affect the toxicity of an unspecified detergent (probably LAS). However, prior exposure to zinc rendered a mixture of detergent and zinc more toxic. Hokanson & Smith (1971) found the presence of suspended particulate matter had no effect on the toxicity of LAS to Lepomis macrochirus, but the lethal threshold concentration was lowered in the presence of sublethal concentrations of fuel oil. Transfer of increased resistance between detergents and other poisons appears unlikely.

An interesting interaction occurs between detergents and some pesticides. Dugan (1967) found that mortality among goldfish exposed to the pesticides Dieldrin and DDT was higher and more rapid among fish previously exposed to detergents. Solon et al. (1969) found that simultaneous exposure of Pimephales promelas to lethal concentrations of Parathion and sublethal concentrations of LAS resulted in a drop of 50% in the 96h LC50 for the pesticide: 1 mg/l LAS caused a reduction from 1.4 to 0.7 mg/l. A concentration of 0.5 mg/l LAS also affected pesticide toxicity, but 0.25 mg/l did not. Results with LAS and DDT were inconclusive, and no interaction was detected between LAS and the pesticide Endrin. Solon & Nair (1970) compared the toxicity of eight phosphate pesticides in the presence and absence of 1 mg/l LAS. Methyl Parathion and Ronnel showed a reduction of approximately 50% in 96h LC50, but Dicapthon was not affected. These three are structurally related to one another and to Parathion. Guthion was not affected, but the 96h LC50 of the related Trithion was reduced by 40%. The 96h LC50

of Trichloronat was reduced by 40%, but that of EPN was unaffected. The most probable explanation of these results is that detergents alter the rate of uptake of the poison by the fish. Non-ionic surfactants have been shown to increase the uptake of barbiturate drugs by Carassius auratus (Linn.), apparently by increasing membrane permeability (Levy & Anello, 1968; Anello & Levy, 1969). The effect persists even after the removal of the fish from the detergent, as may have been the case in Dugan's (1967) experiments, and occurs at low concentrations. Anionic surfactants have not been investigated with respect to fish, but their influence on membrane permeability in non-piscine systems is well established. Gibaldi & Feldman (1970) cite several investigations which showed natural and synthetic surfactants to increase membrane permeability to a variety of substances including barbiturates, salicylic acid, ethanol, thiourea, insulin and antibiotics.

Calamari & Marchetti (1973) have recently investigated the toxicity of mixtures of detergents and metals. In the terminology of Sprague (1970), mixtures of copper and ABS, copper and LAS, and mercury and LAS exerted a more-than-additive toxicity; while a mixture of copper with non-ionic nonylphenol ethoxylate was found to be less-than-additive in toxicity.

Calamari & Marchetti (1973) postulated a mechanism for the increased toxicity of metal-surfactant mixtures, based on the formation of a surfactant-metal-surfactant complex in which a divalent cation substitutes for the sodium ions from two surfactant molecules. This would effectively double the chain length, thus increasing toxicity. This postulate is based on an interaction which is thought to occur between calcium ions and anionic surfactants and was invoked also by Tovell et al. (1974) to explain the higher uptake of sodium lauryl sulphate by fish in hard water than in soft water. In this case, however, the basis of the enhanced detergent effect was thought to be

the "reduced ionic character of this molecular species" facilitating the passage of the calcium-bound SLS across the gill membrane. Further information on the chemistry of this complexation would be highly desirable. Unfortunately, Calamari & Marchetti (1973) did not undertake any experiments in which fish were exposed to metals after pre-treatment with sublethal concentrations of surfactant, so it is not known whether detergents increase the permeability of fish to toxic divalent cations.

1.3 TOXIC EFFECTS OF DETERGENTS

A. Acute effects on fish

Apart from minimal changes in liver and kidney structure (Lang, 1967) of uncertain significance, histological damage to fish resulting from acute detergent poisoning is confined, as far as is known, to the gills and epidermis. Chemoreceptor organs are damaged (Bardach *et al.*, 1965). The pharyngeal wall may show a response, becoming swollen and invested with leucocytes; mucous cells in the gills and pharyngeal wall show increased activity (Brown *et al.*, 1968).

The gill damage has been described by a number of authors (Schmid & Mann, 1961, 1962; Lemke & Mount, 1963; Lang, 1967; Brown *et al.*, 1968). Although accounts differ in detail, histological examination reveals essentially swelling and thickening of the respiratory epithelium, clubbing and adhesion of the secondary lamellae, and eventual breakdown of the gill tissue. Haemorrhage has occasionally been reported. Similar damage is caused by a wide variety of toxicants, but how far the gill damage is a generalised response and what aspects of the response are specific to particular toxicants is not known. It would not be unreasonable to expect gill responses to chemically diverse toxicants to contain specific features related to the mode of action of the poison, either in the nature of the damage at the cellular level, or in the overall sequence of events during the course of gill damage. This thesis is largely concerned with the investigation of this point, and the relevant literature to date is discussed fully in relation to the

results of this investigation in the following chapters.

B. Low-level effects

The effects of low levels of pollutants are usually studied over long exposure periods, but it is wrong generally to assume that such effects can only be detected over long periods. There are several examples of detergents acting sublethally at low concentrations within a relatively short time, and these cases will be considered here together with the more conventional studies of chronic toxicity. The measurement of sublethally toxic concentrations depends on the definition and detection of subtle criteria of toxic effect. Provided these are ecologically meaningful (Sprague, 1971), they will give a more accurate guide to acceptable levels of pollution than tests involving the criterion of death, because they simulate field conditions more closely. There have been several attempts to detect and define suitable criteria with respect to detergent action on fish.

Lemke & Mount (1963) exposed Lepomis macrochirus to concentrations of 3, 6 and 13 mg/l ABS for 30 days in each of three tests. No change occurred in the histological condition of the kidney, spleen or small intestine. Fatty deposition in the liver was ascribed to lack of activity under laboratory conditions. Swimming performance was unaffected, but a reduction in growth was detected in one test conducted during the growing season. Thickening of the gill epithelium and some clubbing of lamellae occurred in the two higher concentrations, but 3 mg/l ABS exerted no detectable effect on any parameter tested. Eisler (1965) found that Fundulus heteroclitus exposed for 150 days to 3 mg/l ABS were not significantly affected regarding length, weight, red blood cell count, gonadosomatic index (100 x gonad wt/body wt), gonadoliver index (liver wt/gonad wt) or somatoliver index (100 x body wt/liver wt). Organs were not examined histologically. Cairns & Scheier (1962) found no correlation between exposure to ABS and the swimming ability of Lepomis gibbosus. Fish were exposed to 5.6

and 18 mg/l ABS for seven weeks. The authors tentatively suggested an increase in oxygen consumption and red blood cell count, although these findings were not conclusive. Both these effects could be the result of gill damage. They reported an increased incidence of rotiferan and protozoan parasites in exposed fish, and suggested that higher mortality in exposed fish indicated stress.

The most striking feature of these reports is the apparent harmlessness of fairly high concentrations. Of course, the factors affecting acute toxicity are likely also to be operative at lower toxicant levels. In addition to any variation thus introduced, errors are likely to arise from the technical difficulties of steadily maintaining low concentrations for protracted periods, and from the limitations of histological methods in detecting and defining damage. Tests with adult fish in isolated, simplified ecosystems are limited also by the absence of the extremes of environmental and biotic conditions through which sublethal toxicity may be expressed at the population level. Pickering & Thatcher (1970) attempted to overcome some of these difficulties by measuring the chronic effects of LAS on Pimephales promelas over one reproductive cycle. Exposure to 2.7 mg/l LAS for five weeks did not affect growth or hatching, but did affect fry survival at 14 days after hatching. Survival, growth, maturity and egg production were unaffected in the two lowest concentrations tested, and the 'no-effect' level was found to lie between 0.6 and 1.2 mg/l.

The alteration of feeding behaviour is a sublethal response of survival significance. Cairns & Loos (1967) found that zebra fish (Brachydanio rerio) exposed to ABS required longer than control fish to consume food dropped into their aquarium. Foster et al. (1966) observed that in Jordanella floridae (Goode & Bean), the visual phase of food finding remained unaffected by exposure to ABS, but treated fish spat out fish as if it were not food. They claimed that the time taken to consume food varied with the concentration of detergent in which the

fish had been maintained, but the few supporting data suggest that this correlation is only approximate. It was suggested that lack of sensory information from taste receptors prevented the fish from recognising food as such, a theory supported by the findings of Bardach et al. (1965) and Vogt (1966) that detergents damage the chemoreceptor organs. Sutterlin et al. (1971) found that concentrations of anionic detergent as low as 1 mg/l can cause reversible blocking of the sensory discharge in the olfactory epithelium of Salmo salar. Sprague & Drury (1969) found that trout in a laboratory channel showed avoidance of ABS at 0.4 mg/l, but were uncertain at 10 mg/l, a nearly-lethal concentration. Korpela (1969) reported similar results for dace and sticklebacks exposed to proprietary detergent formulations. In the field, fish may therefore be unable to detect high concentrations of detergents; and exposure to detergents may make avoidance of other pollutants difficult. Indeed, all functions involving chemoreceptor organs, such as feeding and migration, are likely to be impaired in exposed fish.

Foster et al. (1969) attempted unsuccessfully to develop a behavioural bioassay in which Jordanella floridae were exposed to a sublethal level of ABS. The frequency of occurrence of each of ten characteristic behaviour patterns in the male was measured for fish exposed to 6 mg/l and fish in control tanks. Individual differences in behaviour and differences attributable to the experimental conditions were found to mask any behavioural alterations which may have been due to the detergent. Egg production was, however, drastically reduced in exposed fish.

Although several sublethal effects of detergents on fish have been described, none as yet can be considered a criterion of toxic effect suitable for general use in detergent bioassays. Invertebrate species are, however, better suited to chronic toxicity testing, as well as being of vital importance to fish in the field, and some strikingly low concentrations have been reported as toxic. Surber & Thatcher (1963)

provided a rough guide to the susceptibility of some invertebrate species to ABS, but gave no indication of toxicity values or no-effect levels. Arthur (1970) studied the chronic toxicity of LAS to three species of known importance in the fish food chain. Of special interest is his study of Gammarus pseudolimnaeus over three generations, which led to the suggestion of an acceptable level for this species as low as 0.2 - 0.4 mg/l. For the snail Campeloma decisum, a figure of 0.4 - 1.0 mg/l was suggested on the basis of less extensive tests. Another snail, Physa integra, was not affected as regards survival, growth, reproduction, feeding or mobility over the six-week test period exposed to concentrations up to 4 mg/l. Ninety-six hour LC50's for C. decisum, P. integra and G. pseudolimnaeus were 27, 9 and 7 mg/l respectively. These figures indicate the importance of choosing suitable test species, since the two snails differ as widely in susceptibility as do fish species. More significantly, it appears from these results that the order of susceptibility of species in acute toxicity tests does not necessarily reflect their response to long term exposure.

Swedmark et al. (1971) found marine molluscs and crustaceans to be moderately to highly resistant to LAS, ABS and three other detergents, in terms of their 96h LC50's compared with those of fish. However, the swimming activity of Balanus balanoides larvae was lost after exposure to 1 mg/l LAS for 100h, and in as little as 6h for one non-ionic surfactant at this concentration. Respiratory movements of this and other invertebrates were also affected. Hidu (1965) reported the mean minimum concentration of anionic surfactant (alkyl aryl sulphonates and alkyl sulphate) causing a reduction in growth and survival of clam (Mercenaria mercenaria) larvae and oyster (Crassostrea virginica) larvae was 1.3 mg/l, with a range of 0.1 - 3.0 mg/l. The corresponding concentration for non-ionic polyether alcohols was 2.3 mg/l with a range of 1 - 5 mg/l. Calabrese & Davis (1967) reported that the percentage of fertilised C. virginica eggs developing normally could be

significantly affected by a concentration of LAS as low as 0.025 mg/l. Granmo (1972) reported that the development of the transitory stages in the life cycle of the mussel Mytilus edulis could be delayed by a concentration of LAS of 0.3 mg/l; larval growth was reduced at concentrations above 0.1 mg/l; and fertility was affected above 0.05 mg/l.

Clearly, invertebrates must be regarded as being at least as sensitive as fish to detergents. If fish bioassays could be conducted over several generations with equal facility, sublethally toxic concentrations would probably be found to be lower than at present.

1.4 MODE OF ACTION OF DETERGENTS ON FISH

There is a gradation in meaning of the term 'mode of action' ranging from the mere description of symptoms at progressively lower levels of integration to the definition of the primary lesion at the biochemical, sub-molecular level. Pharmacologists use the phrase strictly in its latter sense, but this discussion adopts a less rigorous interpretation. (This point is discussed more fully in Chapter 6).

The immediate cause of death from acute detergent poisoning where extensive gill damage occurs is likely to be either asphyxiation or loss of osmotic or ionic stability. Skidmore (1970) showed that death in trout with gills damaged by exposure to zinc was due to asphyxia rather than osmoregulatory imbalance. Cairns & Scheier (1966) kept Lepomis gibbosus for periods of 42 days in concentrations of ABS sufficient to cause extensive gill damage, but treated fish and control fish maintained similar blood chloride levels in external chloride concentrations of 60 and 6,500 mg/l. Since the detergent concentration was far below an acutely lethal one, it is possible that homeostatic mechanisms were not seriously affected. The extent and nature of gill damage in relation to the survival time of the fish has not been studied for any pollutant. Asphyxiation due to gill damage is almost certainly not the only mechanism of acute poisoning, but alternative actions have not been

investigated. Moreover, although zinc was shown by Skidmore (1970) not to be a rapid internal poison, this has not been shown for detergents. It is not so far known whether detergents enter fish, and to what extent they exert toxic action internally. Therefore, although gill damage may occur as a result of detergent poisoning, it remains to be established that death is caused primarily by loss of any gill function and not by some form of internal poisoning to which gill damage may be only a contributory or complicating factor. Concentrations regarded as acutely lethal may act over several days, or within a few minutes (Sprague, 1969). Over such a range of survival times it is unsafe to assume a single mode of action. Chapters 3 and 4 describe an investigation of this point in relation to gill damage.

It is difficult to determine which of the long-term effects resulting from sublethal exposure to detergents are direct, and which simply reflect physiological adaptations to sublethal stress. Histological changes in internal organs, for instance, probably fall into the latter category. Similarly a reduced growth rate may be mediated by impaired feeding owing to chemoreceptor damage; from an increased metabolic cost of respiration as a result of gill damage; by an unknown environmental factor; or by the interaction of an environmental factor and a toxic effect, an occurrence which would be unmonitored in a control experiment. In other words, observed sublethal toxic effects may occur at a level of integration so far removed from the primary effect that no inferences as to the mode of action of the poison may be drawn from them.

The mechanisms by which detergents cause their observed effects are not certainly known. Toxic effects of detergents on living organisms have been studied since the 1930's, largely with regard to bacteriocides. Much research has therefore been carried out on detergent-protein interactions, early work along these lines being reviewed by Putnam (1948). It is established that proteins can be altered, reversibly or irreversibly, by detergents in low concentrations; indeed, the use of detergents as

protein denaturants is a widely-known technique in biochemical research. At higher concentrations, when the detergent is present largely in the form of micelles, detergents have the property of solubilising organic material. Most toxicological research is carried out at concentrations too low for the formation of micelles to occur, but even below the critical micelle concentration, detergents probably have more than one mode of action. At low concentrations, detergents alter membrane permeability, as has already been discussed. At higher concentrations, detergents undoubtedly exert a disruptive effect. Acute gill damage is a likely example of this, and Goldacre's (1968) demonstration of the effects of detergents on the cell membrane of Amoeba provides further confirmation. The interaction of detergents with proteins also indicates the likelihood of their having widespread systemic toxic effects internally, should they find their way into fish. It should be pointed out that the interaction of non-ionic detergents with proteins is not well understood, although some interaction is thought to occur (Swisher, 1970).

The role of surface tension reduction in detergent toxicity has excited controversy. Prat & Giraud (1964) accept uncritically the notion that detergent toxicity is due to surface tension reduction alone. They write " ..... It is difficult to believe that substances of widely different chemical composition like lauryl sulphate and dodecylbenzene sulphate would be toxic in doses which are (so) ..... close if they worked by chemical action. It may, therefore, be accepted that the action of surface-active agents on fish is the purely physical one of reduced surface tension." These authors have ignored entirely the overwhelming biochemical evidence of both temporary and permanent alteration of proteins by interaction with detergents; and in any case, the substances mentioned can hardly be regarded as being "widely different", at least in this context. They go on to describe respiratory exchange as "an osmosis phenomenon", though it is of course more

correctly regarded as a process of diffusion, and suggest that symptoms of asphyxia indicate that "..... the fish fights, consciously or subconsciously, to raise the surface tension of water when it passes over the gills." It need hardly be pointed out that fish asphyxiate when poisoned by non-surface-active substances.

Nevertheless, there have been attempts to investigate this curious theory. Bock (1965) reported that a range of natural and synthetic surfactants damaged the gills of fish at concentrations which reduced the surface tension to 50 dyn/cm. However, he failed to show that the damage was in all cases comparable, and that it did not occur at lower concentrations. This approach was also unsound because it ignored the possibility of toxic action occurring other than on the gills. Marchetti (1965a) reported, on the basis of 6h LC50's for Carassius auratus exposed to a large number of detergents, that toxic concentrations were not such as to lower surface tension uniformly. A similar experiment (Calamari & Marchetti 1973) showed that the 14 day LC50's of ABS, nonylphenol ethoxylate and LAS produced a surface tension in solution of 45, 50 and 67 dyn/cm respectively. These results strongly indicate that a simple relationship between surface tension and toxicity does not exist. The toxicity of detergents to bacteria has been investigated more fully in this respect, and it is clear that surface tension is not related causally to toxic effect (Rahn, 1945; Glassman, 1948).

That surface activity is nonetheless important may be inferred from the fact that detergents in homologous series differ in toxicity, frequently showing a clear relationship between structure and activity. Thus for the common anionic detergents, surface activity increases and solubility decreases with increasing chain length, resulting in a maximum in the range C_{12} to C_{16} for both detergency (Price, 1945) and toxicity (Fig. 1.1). A toxicant must contact the fish in order to exert its effect, and a greater tendency to concentrate at interfaces must

make the detergent more likely to do this. The toxicity of a detergent will therefore be a function of both its surface activity and its chemical toxicity. Albert (1965, p.325), discussing antibacterial surface-active agents, draws a similar conclusion: "All three classes of substances are surface active, but this property is not enough in itself to cause biological action. The three charge-types of surface-active substances, the cationic, anionic and neutral, are found to be highly antibacterial, slightly antibacterial, and inert, respectively, when examples of equal surface activity are compared. Thus high surface activity does not explain why substances cause damage, but only how they become concentrated on the bacterial surface". It is also conceivable that a reduced surface tension may modify the sequence of events during, for example, the disruption of gill epithelium in acute detergent poisoning; but there is no basis for the belief that surface tension reduction causes the disruption.

The comparative toxicity of detergents may hold important clues to their mode of action, although this possibility largely awaits investigation. In addition to the work on fish already mentioned, it is known that for Daphnia magna (Freeman, 1953), the planarian Dugesia lugubris (Saski et al., 1971) and bacteria (Putnam, 1948; Glassman, 1948), different detergents differ widely in toxicity. This approach should help to indicate the relative importance of the physical and chemical aspects of detergent toxicity.

1.5 TOLERANCE LIMITS

Lloyd (1972) has pointed out the difficulties of establishing valid water quality criteria. Of overriding importance with respect to detergents is the fact that detergents in rivers will be partially degraded, and therefore toxicologically dissimilar from raw detergent. Data from field measurements may be unreliable because of the limitations of analytical methods, because of the presence of other pollutants, or because the pollutant levels and other environmental conditions will

fluctuate. Such factors as the effects of detergents on the re-aeration of streams (Gameson et al., 1956), though difficult to assess, may be of practical importance. While it is unlikely that any single standard will be appropriate for all bodies of water, it is established practice generally to recommend pollutant levels which should not be exceeded. Existing standards are 0.5 mg/l ABS in drinking water supplies (U.S Public Health Service and World Health Organisation) and 1 mg/l ABS in other waters (W.H.O.). No revision has been made since the introduction of soft detergents, although these are known to be more toxic in the laboratory.

Although detergent concentrations in natural waters have fallen conspicuously, it has never been established how far the rapid biodegradability of soft detergents compensates for their greater toxicity, a reflection of the fact that they were introduced not to preserve aquatic life but to reduce foaming in sewage works and water courses. Even if recommended criteria were adhered to, it is not necessarily the case that the same standards will suffice for soft as for hard detergents. In the field, however, it is degraded detergents which are of prime concern, and the degradation products of soft and hard detergents are probably similar. The toxicity to fish of partially-degraded detergents has been reported on only two occasions (Herbert et al., 1957; Swisher et al.,1964), in each case being found considerably less than that of raw detergent. But until the toxicity of degraded and partially-degraded detergent has been more extensively studied, the meaningful application of laboratory-determined data to field situations will remain difficult. In addition, the relationship between the toxicity of detergents and their response to chemical analyses should be studied, especially during the course of degradation.

The concept of an application factor, whereby the results of acute toxicity tests are modified to give a 'safe' concentration, has not, therefore, been validated with respect to detergents. Two studies have

proposed application factors. Pickering & Thatcher (1970) studied the effects of low-level exposure to LAS on Pimephales promelas through one reproductive cycle, and detected no toxic effect at 0.6 mg/l. Comparison of this figure with the results of acute toxicity tests on fingerlings led them to propose an application factor of between 0.14 and 0.28. Hokanson & Smith (1971) compared the toxicity of LAS to Lepomis macrochirus fingerlings with the concentration of LAS causing no mortality among the most sensitive life stage, namely sac-fry. They proposed an application factor of 0.47. Neither of these figures can be recommended for general use.

In addition to fish and their food organisms, primary productivity of waters may also be influenced by detergents. Paradoxically, the most conspicuous effect of detergents on primary productivity has been to increase it to such an extent as to cause accelerated eutrophication. This effect is due to the phosphate additives rather than the detergents themselves, and is outside the scope of the present discussion. The surfactant ingredients actually reduce the growth of diatoms when tested alone, this effect becoming detectable as low as 0.3 mg/l ABS for Nitzschia linearis in soft water. Under these conditions, 1 mg/l ABS was found to reduce growth by 20% (Cairns et al., 1964). Navicula seminulum in hard water, however, was not affected by 10 mg/l ABS. It is surprising that the effects of detergents on aquatic microphytes have not been more fully investigated.

There seems to be no indication from the toxicological evidence that the present standards, where maintained, are generally inadequate. From the point of view of the preservation of aquatic life, however, it is absurd to discriminate between water to be used for drinking and other waters. Therefore the extension of the lower limit (0.5 mg/l) to cover all waters seems to be a desirable step, especially in the light of recent findings which indicate invertebrates to be rather more sensitive than was formerly thought. But as Lemke & Mount (1963) put it,

the significance of the effects of a given level of pollution "varies with one's philosophy of stream use."

1.6 SUMMARY AND CONCLUSIONS

Detergents are acutely toxic to fish in concentrations reportedly lying between 0.4 mg/l and about 40 mg/l. The most important environmental factors influencing toxicity are the type of detergent and its molecular configuration, and the dissolved oxygen concentration. Important biotic factors are the species, age and life stage of the fish, and acclimation. Factors of less well-defined importance are water hardness, temperature and salinity. Exposure to low levels of detergents prior to or simultaneously with exposure to some other poisons, notably pesticides, lowers resistance to these poisons. Transfer of increased resistance between detergents and other poisons appears unlikely.

The acute toxic effects of detergents on fish include severe gill damage, destruction of chemoreceptor organs and damage to the epidermis and pharyngeal wall. Low concentrations may cause functional impairment of chemoreceptors. The immediate cause of death where gill damage occurs extensively may be asphyxia, but acutely toxic actions not involving gill damage have not been investigated and it is not known whether detergents are internal poisons. Chronic effects include reduction in growth, egg production and fry survival and alteration of feeding behaviour. However, chronic toxicity testing with fish as a routine method of detergent bioassay has not so far been shown to be capable of producing reliable and reproducible results. Sublethal responses of invertebrates have been reliably assessed in very low concentrations, in some cases below 0.1 mg/l.

Detergents probably have more than one mode of action, likely ones being denaturation of proteins and the alteration of membrane permeability and transport characteristics. Surface tension reduction is

not in itself a cause of toxicity.

As released to the environment, detergents are usually partially degraded. There has been no extensive study of the comparative toxicology of raw and partially degraded detergent, so the extrapolation from laboratory data to water quality criteria is more than usually difficult for detergents. Existing standards, when adhered to, are probably largely sufficient, but the greater toxicity of soft detergents, in spite of their rapid biodegradability, and the very low concentrations reported as toxic to some invertebrates, indicate that some waters may require special consideration. The extended application of the 0.5 mg/l standard is recommended.

TABLE 1.1 Acute toxicity of detergents to various species under different conditions.

These values are summarised from the original papers, and are a representative rather than an exhaustive list. Some results are discussed in more detail in the text, and all are more fully presented in the original reports.

DETERGENT	TEST FISH	RESULT	COMMENT	REFERENCE
ABS	Salmo gairdneri	10 day LC50 5 mg/l.	No threshold detected in 12 week test.	Herbert et. al., 1957
"	Pimephales promelas	96h LC50 between 3 and 9 mg/l.	Several samples tested. More toxic in hard water.	Henderson et. al., 1959
"	Lepomis macrochirus	96h LC50 17 mg/l.	No effect due to water hardness	Cairns & Scheier, 1962
"	Lepomis gibbosus	" 22 mg/l.	Only tested in soft water	"
"	Fundulus heteroclitus	96h LC50 6.8 mg/l.	Estuarine species, salinity 24°/oo	Eisler, 1965
"	Mugil cephalus	" 3.1 mg/l.	" " " "	"
"	Pseudopleuronectes americanus	" 2.5 mg/l.	" " " "	"
"	Anguilla rostrata	" 2.3 mg/l.	" " " "	"
"	Menidia menidia	" 2.1 mg/l.	" " " "	"
"	Notropis atherinoides	" 7.4 mg/l.	Species arranged in descending order of susceptibility	Thatcher, 1966

TABLE 1.1 Continued

DETERGENT	TEST FISH	RESULT	COMMENT	REFERENCE
ABS	Pimephales notatus	96h LC50 7.7 mg/l.		Thatcher, 1966
"	Lepomis macrochirus	" 8.2 mg/l.		"
"	Campostoma anomalum	" 8.9 mg/l.		"
"	Notropis stramineus	" 9.0 mg/l.		"
"	Erycymba buccata	" 9.2 mg/l.		"
"	Notropis ardens	" 9.5 mg/l.		"
"	Pimephales promelas	" 11.3 mg/l.		"
"	Notropis cornutus	" 17.0 mg/l.		"
"	Cyprinis carpio	" 18.0 mg/l.		"
"	Ictalurus melas	" 22.0 mg/l.		"
"	Gadus morrhua	96h LC50 3.5 mg/l.	Marine species	Swedmark et. al., 1971
"	Pleuronectes flesus	" 6.5 mg/l.	" "	"
"	Salmo gairdneri	14 day LC50 10.7 mg/l.		Calamari & Marchetti, 1973
LAS	Lepomis macrochirus	96h LC50 3 mg/l.	Carbon chain 12 atoms long	Swisher et al., 1964

TABLE 1.1 Continued

DETERGENT	TEST FISH	RESULT	COMMENT	REFERENCE
LAS	Lepomis macrochirus	96h LC50 0.6 mg/l.	Carbon chain 14 atoms long	Swisher et. al., 1964
"	Notropis atherinoides	96h LC50 3.3 mg/l.	Species arranged in descending order of susceptibility	Thatcher & Santner 1967
"	Lepomis macrochirus	" 4.0 mg/l.		"
"	Pimephales promelas	" 4.2 mg/l.		"
"	Notropis cornutus	" 4.9 mg/l.		"
"	Ictalurus melas	" 6.4 mg/l.		"
"	Lepomis macrochirus	Lethal threshold concn. 1.6 - 3.1 mg/l.	Tested under various conditions	Hokanson & Smith, 1971
"	Gadus morrhua	96h LC50 1 mg/l.	Marine species 96h LC50 decreases with increased temperature	Swedmark et. al., 1971
"	Pleuronectes flesus	" 1.5 mg/l.	"	"
"	Salmo gairdneri	14 day LC50 1.7 mg/l.		Calamari & Marchetti, 1973
Alkyl sulphate	Pimephales promelas	96h LC50 5-6 mg/l.	Slightly more toxic in hard water	Henderson et. al., 1959

TABLE 1.1 Continued

DETERGENT	TEST FISH	RESULT	COMMENT	REFERENCE
Polyoxyethylene ether	Salmo salar	Lethal threshold conen. 2.5 mg/l.	Non-ionic. 4 units ethylene oxide per molecule	Wildish, 1972
"	"	lethal threshold conen. 37 mg/l.	Non-ionic. 23 units ethylene oxide per molecule	"
Polyoxyethylene ester	Lepomis macro-chirus	96h LC50 37 mg/l.	Type not specified. Unaffected by hardness. Non-ionic	Henderson et al., 1959
"	Salmo salar	Lethal threshold conen. 22 mg/l.	Polyoxythylene (14) monolaurate Non-ionic	Wildish, 1972
Nonylphenol ethoxylate	Salmo gairdneri	14 day LC50 4.3 mg/l.	Non-ionic. 8 units ethylene oxide per molecule	Calamari & Marchetti, 1973

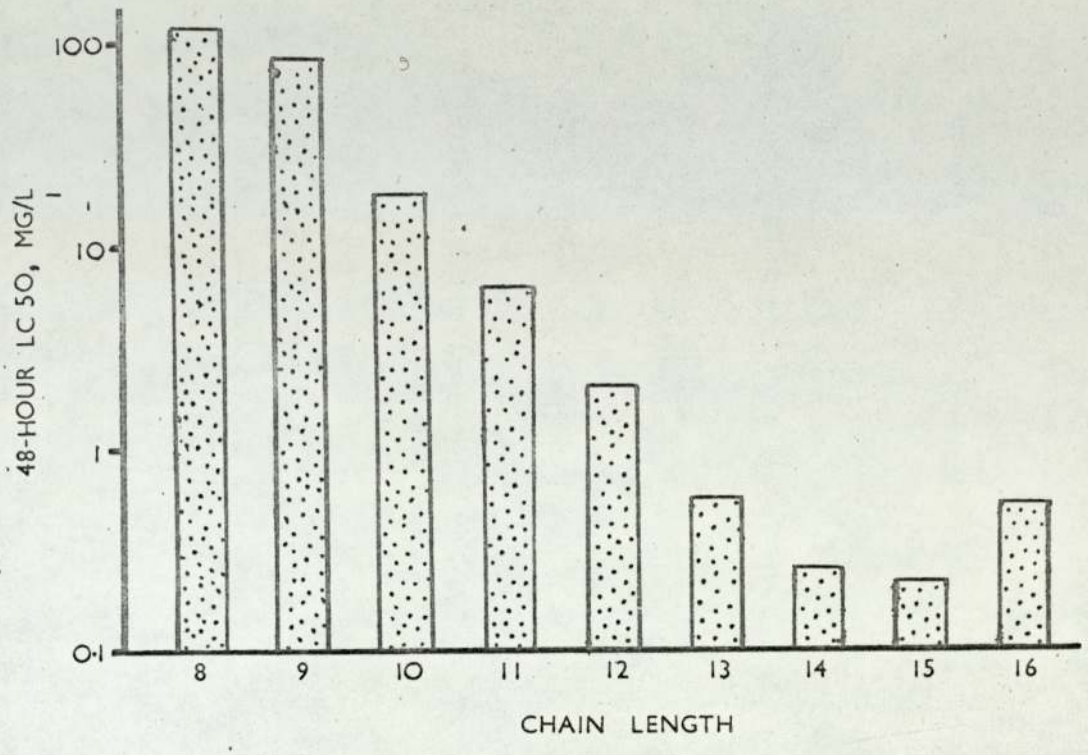


Fig. 1.1. Diagram showing the influence of chain length on detergent toxicity. The 48 h LC50 of LAS to Idus idus (Linn.) is plotted on a logarithmic scale against the number of carbon atoms in the hydrocarbon chain. Data from Hirsch (1963).

CHAPTER 2. Toxic effects of an acutely lethal concentration of the anionic detergent sodium lauryl sulphate (SLS) on the gills of rainbow trout.

2.1 INTRODUCTION

Many toxic agents are known to damage fish gills in superficially similar ways, but it may be expected that more discerning studies will demonstrate reactions diagnostic to some toxicants, possibly related to their specific modes of toxic action. Elements common to all pathological reactions are also of interest to extend knowledge of gill function under normal and abnormal conditions. This paper describes the changes induced in the gills of rainbow trout, Salmo gairdneri (Richardson), on exposure of the fish to an anionic detergent, sodium lauryl sulphate (SLS), at a concentration of 100 mg/l. The chemical dissimilarity of this toxicant from zinc sulphate, the toxicant employed in a similar study by Skidmore & Tovell (1972), also using rainbow trout, raises the possibility of detecting diagnostically different features of its toxic effects. Survival times were similar in the two studies. Thus toxic effects of SLS and zinc may be directly compared using the same species under similar conditions. There is a fairly extensive literature on the toxicity of detergents to fish and the occurrence of gill damage in fish exposed to detergents is well known (Abel, 1974). Salmo gairdneri gills are of typical teleost form, and have recently been described in detail by Morgan & Tovell (1973).

2.2 MATERIALS AND METHODS

Yearling Salmo gairdneri, mean weight 27g (s=7.6g), mean fork length 13 cm (s=1.6 cm) were obtained from Packington Fishery, Meriden, Warwickshire and acclimated to Birmingham tap water at 15°C for 7 days. The fish were fed daily on pelleted food until the day before experiment. Experiments were conducted in Birmingham tap water (total hardness 25 mg/l as $CaCO_3$; pH 7.6) at 15°C in polythene tanks containing 100 litres of test solution, aerated continuously. The toxicant, sodium lauryl

sulphate, $CH_3(CH_2)_{11}.OSO_3Na$, containing not more than 1 per cent C_{10} and C_{14} alcohol sulphates, was supplied by British Drug Houses Ltd.

Ten fish were exposed to 100 mg/l SLS. Individual survival times were noted, death being defined as permanent opercular immobilisation, and cumulative percentage mortality was plotted on a probit scale against elapsed time on a logarithmic scale (Bliss, 1937). Median survival time (LT50) and its 95% confidence limits were estimated from the resulting line by the graphical method of Litchfield (1949).

Subsequently 25 fish were exposed to 100 mg/l SLS. Gill tissue was collected for examination after 1½ h exposure (30% LT50), 3 h exposure (60% LT50), at initial surfacing, overturn (fish lying on side at bottom of tank) and opercular immobilisation. Five fish were sampled at each stage. After decapitation of the fish, gill samples were removed by clipping the tips of the filaments of the second gill arch on the left side. From two fish exposed for 3 h, gill samples were also taken from the first and third arches of the right side, and the fourth arch of the left side.

Tissue was fixed in 2.5 per cent glutaraldehyde in 0.1 M sodium cacodylate (pH 7.2 - 7.4) at 0°C for 1 h. (Sabatini et al., 1963). It was washed overnight in buffer alone and post-fixed in 1 per cent osmium tetroxide in 0.13 M sodium cacodylate for 1 h at 4°C. Tissue was then stained in 1 per cent aqueous uranyl acetate (Watson, 1958), dehydrated in alcohol and epoxypropane and embedded in Araldite. Sections were cut on an LKB Ultrotome II. Thick sections (0.5 - 1.0μ) were mounted on glass slides and stained with hot alkaline methanolic methylene blue solution for light microscopical examination. Ultra-thin sections, mounted on copper grids, were stained in lead citrate solution (Reynolds, 1963) and examined in an AEI EM6B electron microscope.

Detergent concentrations were measured before and after the experiments by the methylene blue method of Degens et al. (1953) using a calibration curve constructed specifically for SLS in a Beckman DB600

spectrophotometer at 650 mμ (100 mg/l SLS produces the same response as 125 mg/l Manoxol OT). No significant decrease in detergent concentration occurred during the experiments.

2.3 RESULTS

Fish first responded to the toxicant by an immediate increase in swimming activity and in coughing rate compared with control fish. Later behavioural responses were surfacing, loss of balance, overturn with loss of mobility and finally death. The graph of cumulative mortality against survival time is shown in Fig. 1.1, the LT50 being 4.9 h (95% confidence limits 3.9 - 6.1 h).

A. Normal gill structure

Figures 2.2 and 2.6 show light and electron micrographs of control trout gill with secondary lamellae in transverse section. The lamellae arise at right angles from both sides of the gill filaments. The epithelium over the lamellae is a double layer of cells, containing scattered chloride and mucous cells. The innermost layer of epithelial cells is closely apposed to the underlying basement membrane, but the outer layer is only loosely attached. Desmosomal connections occur between the cells within each layer but not between layers, so that the epithelium contains some intercellular lymph spaces between layers, although in well-fixed tissue these are not prominent. The epithelium over the filaments is several cells deep, closely apposed to the underlying tissue and continuous with the lamellar epithelium.

Beneath the epithelium lies a collagenous basement membrane, whose inner element is continuous with the supporting columns of the pillar cells of the secondary lamellae. The flanges of the pillar cells form the endothelium of the lamellar blood spaces, except around the distal edge of the lamella where the marginal channel is partly enclosed by separate endothelial cells. Within the filament lies the filamental sinus, lined with connective tissue and containing blood and lymph

vessels plus a few leucocytes.

B. Changes induced by sodium lauryl sulphate

Gills of fish exposed to 100 mg/l SLS for 1½ h (30% LT50) showed four main signs of damage.

a. Secondary lamellae were disarrayed in about half the samples examined (Fig. 2.3). Intercellular spaces in the lamellar epithelium were larger and more numerous than in controls, and the epithelium appeared slightly swollen.

b. The filament epithelium was also swollen and contained extensive, intercellular lymphoid spaces (Fig. 2.3) frequently occupied by leucocytes.

c. Many chloride cells seen in light microscrope sections had conspicuous pyknotic nuclei, and cytoplasmic staining was consistently fainter than in control tissue. Electron microscopical examination revealed that these cells were undergoing death and disintegration (Fig 2.12). A few unspecialised epithelial cells had undergone generally similar changes, and pyknotic nuclei occurred extensively in filament cartilage cells. These changes appeared to be associated with and preceded by the development of granular endoplasmic reticulum and lysosomes (Fig. 2.11).

d. There was slight cellular debris between lamellae, apparently from the sloughing of dead epithelial cells (Fig. 2.9). Generally, the final stages of cellular disintegration occurred after cells became detached.

Gills of fish exposed for 3 h (60% LT50) were generally similar to those of fish exposed for 1½ h, though many sections showed the damage in a more advanced state. In a few lamellae the marginal channel was enlarged, although in these lamellae the lamellar blood space was generally reduced. In a small number of lamellae some or all of the blood spaces were completely occluded. Intercellular lymph spaces in

the filament epithelium were invaded by both lymphocytes and granulocytes (Fig. 2.7), and the filament epithelium had lifted from the underlying tissue in some areas of the gills. An abnormally large number of active eosinophilic leucocytes were present in the circulating blood (Fig. 2.11).

The stages of surfacing, overturn and death were each marked by a progressive deterioration in the condition of the gills (Figs. 2.3, 2.4, 2.5). Gradually the filament and lamellar epithelium became raised, while the pillar cell system increasingly collapsed. This collapse appeared to progress from the proximal to the distal regions of the lamella, the proximal and marginal channels alone remaining open. Reduction of the blood spaces continued, the pillar cell flanges occupying most of the available space, until the individual pillar cell blood spaces became completely blocked to the passage of blood cells (Fig. 2.8). Concurrently, pillar cell bodies became reduced to about half their normal size. Collapse of the pillar cell system was a good indicator of the progress of gill damage, the percentage of affected secondary lamellae increasing with exposure (Table 2.1). At death, the filament and lamellar epitthelium was completely raised and lay across the lamellar margin over extensive areas of the gills; elsewhere the epithelium was swollen and completely detached from the pillar cell system. Virtually all lamellar blood spaces were contracted or occluded, and haematomas were common (Figs. 2.4, 2.5, 2.10). At no time, however, did the epithelium appear to rupture. Though in places very thin and stretched, the epithelial barrier remained unbroken and its desmosomes intact. Erythrocytes, although leaking from within the pillar cell system were apparently confined within the intact outer layer of the lamellar epithelium (Fig. 2.10). The large quantity of cellular debris between lamellae indicated that many cells had been sloughed off but this was apparently achieved without puncturing the lamellar epithelium (Fig. 2.9). Intact mucous cells were numerous even in fish sampled at death; there was no evidence of an abnormally high rate of mucus discharge.

During the later stages of poisoning, small numbers of erythrocytes could be observed in the filamental sinus in some samples.

This overall pattern of damage was subject to some variation. After 1½ h exposure some samples were indistinguishable from control tissue while others showed some or all of the changes noted. Reactions of adjacent secondary lamellae varied widely. However, after 3 h exposure no region of the gills was entirely unaffected.

2.4 DISCUSSION

Previous studies have suggested that the reactions of fish gills to acutely toxic concentrations of pollutants are generally non-specific. With regard to detergents, Schmid & Mann (1961, 1962) and Brown *et al.* (1968) reported damage caused by alkylbenzene sulphonates (ABS) to *Salmo gairdneri* gills as swelling of epithelial cells, adhesion of secondary lamellae, detachment of epithelium from the pillar cell system, discharge of mucous cells and haematomas. Lang (1967) reported detachment and eventual destruction of the epithelium of *Carassius auratus* gills exposed to ABS and SLS. Generally similar effects are produced by heavy metal salts (Schweiger, 1957; Haider, 1964; Skidmore & Tovell, 1972), nitrophenols (Christie & Battle, 1963), phenol (Mitrovic *et al.*, 1968), phenylmercuric hydroxide (Lindahl & Hell, 1970) and formaldehyde (Smith & Piper, 1972), although the discharge of mucous cells has not been universally observed.

Another commonly reported non-specific feature is leucocytic infiltration, associated with the oedematous detachment of the epithelium as in a typical mammalian inflammatory response (Anderson, 1966). This feature is demonstrated in the present study and has been previously reported by Brown *et al.* (1968), Mitrovic *et al.* (1968), Skidmore & Tovell (1972) and Smith & Piper (1972) in trout exposed to a detergent, phenol, zinc and formaldehyde respectively. Inflammation has not been reported in all studies of gill damage, possibly because it does not occur at all acutely-toxic concentrations of pollutants. Brown *et al.*,

(1968) found some evidence of this using two concentrations of detergent.

Several authors have reported features of gill damage not common to all pollutants. Mitrovic et al. (1968) reported necrosis in gill and pharyngeal epithelium and the erosion of gill filament tips in trout exposed to phenol. Smith & Piper (1972) observed nuclear swelling, pyknosis and fragmentation in the gills of trout exposed to formaldehyde. In an electron microscopical study of the effects of zinc on trout gills, Skidmore & Tovell (1972) found that although the gill reaction was primarily an inflammatory response, the chloride cells became detached early in a characteristic and prominent manner, and the pillar cells became vacuolated. The present study suggests that a concentration of SLS lethal over a similar period caused a similar gill reaction, but without pillar cell vacuolation; and chloride cells did not detach any more readily than unspecialised epithelial cells.

A striking feature of SLS-induced damage, apparently not shared in general with other poisons, is the extensive evidence of cellular death (nuclear pyknosis, formation of granular endoplasmic reticulum and lysosomes, cytoplasmic disintegration and the widespread sloughing of cells) at an early stage. It was noted that dying chloride cells lose their microvilli (Fig. 2.12) an event previously observed by Olson & Fromm (1973) in their scanning EM study of trout gills. The discharge of mucous cells reported by earlier authors (Schmid & Mann, 1961, 1962; Brown et al., 1968) was not observed with SLS.

It is implicit in the search for diagnostic pathological reactions that such features must be related to the mode of action of the poison. Gill responses may be a rewarding source of information in this respect, since gill structure is well known and deviations from the normal should be easy to detect. Unfortunately, the action of too few toxicants has been studied in detail but it is possible to distinguish between SLS and zinc at the concentrations tested, in spite of the close similarity of the overall reaction: zinc does not produce the signs of cellular death

referred to above, and SLS does not cause pillar cell vacuolation or selective detachment of chloride cells. It must not be expected, however, that these distinguishing reactions will occur at all concentrations. Light microscope studies of the effects of phenol (Mitrovic et al., 1968) and formaldehyde (Smith & Piper, 1972) indicate that these poisons may share with SLS the capacity to induce extensive cellular death from the earliest stages of poisoning. This may be expected from the known chemical properties of the three substances, all of which bind readily to proteins and other cell constituents. Differences in toxic effect at the cellular level between these poisons and, say, heavy metal ions may be related to the chemical nature of the interactions which occur between the toxicant and the living material.

This study has further demonstrated that the gill response to pollutants is at least partially non-specific, and inflammatory at the concentration and survival times tested. These aspects of the response are worthy of further study, particularly in relation to inflammation produced by disease or physical stresses, since the immune responses of fish are not well understood. It has been suggested (Morgan & Tovell, 1973) that epithelial lifting may exert a protective effect, hindering pollutant uptake by increasing diffusion distance. Clearly, this response will take place at the expense of respiratory efficiency, but such a response could well occur in the zones of physiological impairment regarded by Lloyd (1972) as 'compensation' or 'breakdown'. Ultimately, however, the respiratory handicap imposed by epithelial lifting must outweigh any protective effect against pollutant uptake. Certainly in the later stages of acute poisoning, especially when asphyxia is the immediate cause of death (Skidmore, 1970) this is likely to be the case. Non-functioning cells in the outer epithelial layer must be removed and replaced if ionic and osmotic stability are to be maintained. Such replacement certainly occurs during SLS poisoning (Fig. 2.9). If many cells are lost by pollutant action, the resulting reorganisation demands

that the total exposed gill area be reduced. This can only be achieved by the raising of the epithelium between the secondary lamellae. With both SLS and zinc sulphate poisoning the unchanged size of cells in the subepithelial spaces indicates that an osmotically normal internal environment is maintained (Compare Fig. 2.7 with Skidmore & Tovell, 1972, Fig. 9). The pattern of lamellar haematomas indicates that the integrity of the outer epithelial layers is upheld even in the final stages of poisoning, and that the resistance of the pillar cell system to disruption may not be as great, relative to that of the epithelium, as is commonly supposed.

This study therefore raises some questions concerning the normal and abnormal physiology of fish: whether fish are more resistant to internal hypoxia than to internal osmoregulatory stress; whether or not osmoregulatory stress manifests itself before respiratory stress; and the implications for the fish of the loss of cellular function at its main interface with its external environment.

TABLE 2.1 Progress of gill damage as indicated by the proportion of secondary lamellae in which some or all of the pillar cell blood spaces are blocked.

Exposure	Number of lamellae examined	Number of lamellae affected	% of lamellae affected
Control	103	0	0
30% LT50	261	2	0.8
60% LT50	342	21	6.1
Surface	197	21	10.7
Overturn	180	137	76.1
Death	267	232	86.9

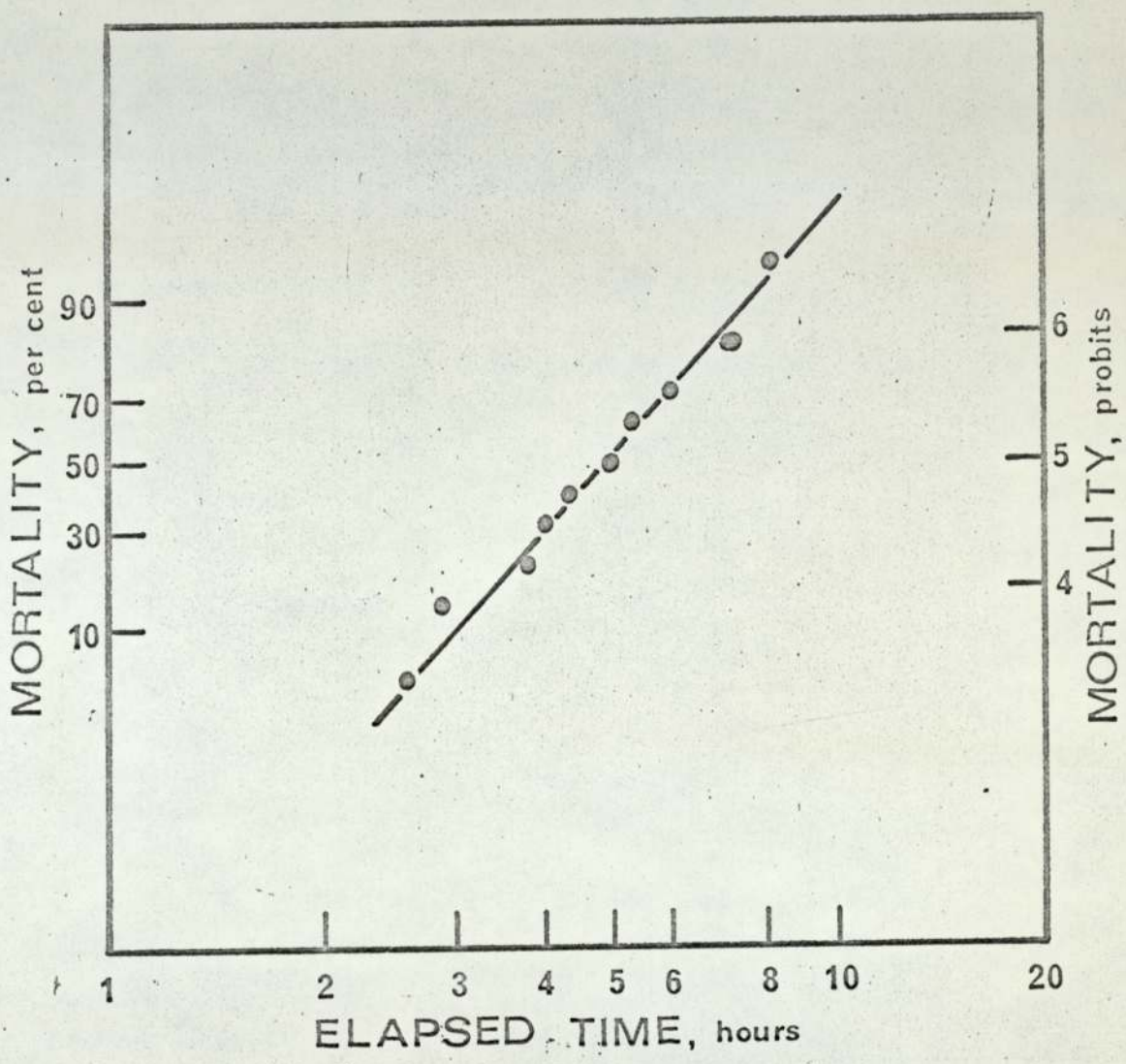


Fig. 2.1. Graph of cumulative mortality (probit scale) against survival time (logarithmic scale) for Salmo gairdneri exposed to 100 mg/l SLS.

Fig.2.2. Light micrograph of a gill filament from a control fish, showing the secondary lamellae in transverse section. Note the regular lamellar array and compact filament epithelium (x 200).

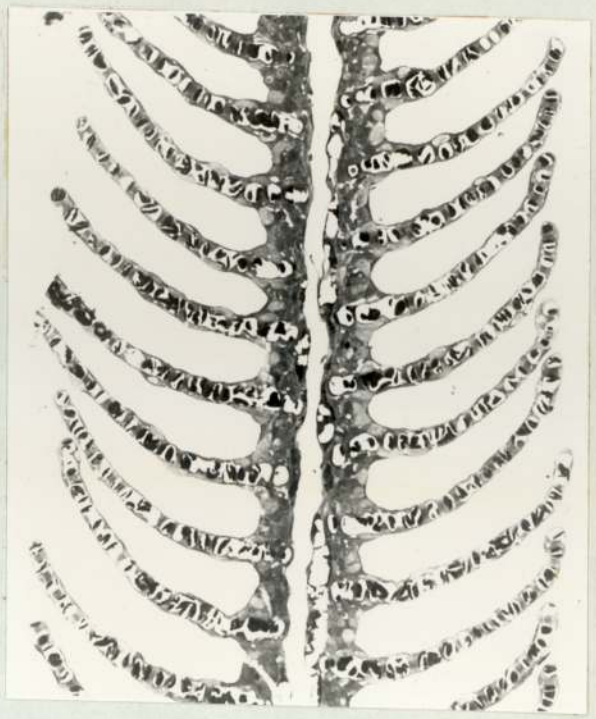


Fig.2.3. Gill section from fish exposed to 100 mg/l SLS for 30% LT50. Secondary lamellae are disarrayed and extensive vacuolation of the filament epithelium has occurred, together with some detachment of lamellar epithelium (x 200).

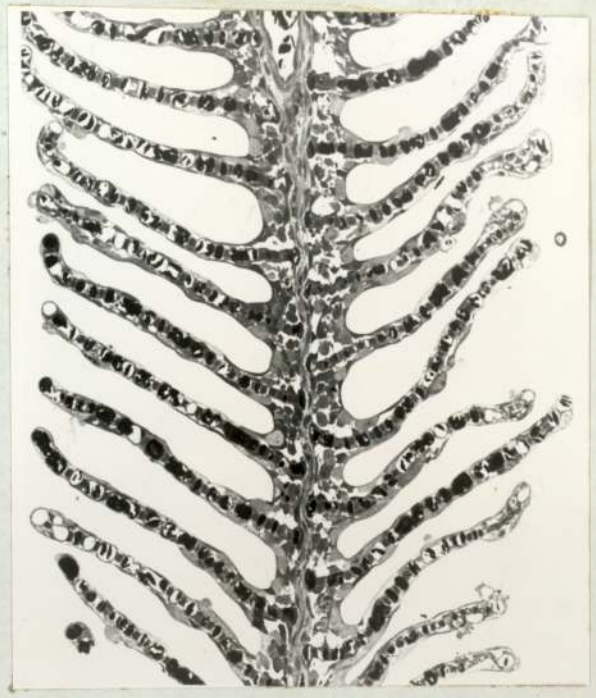
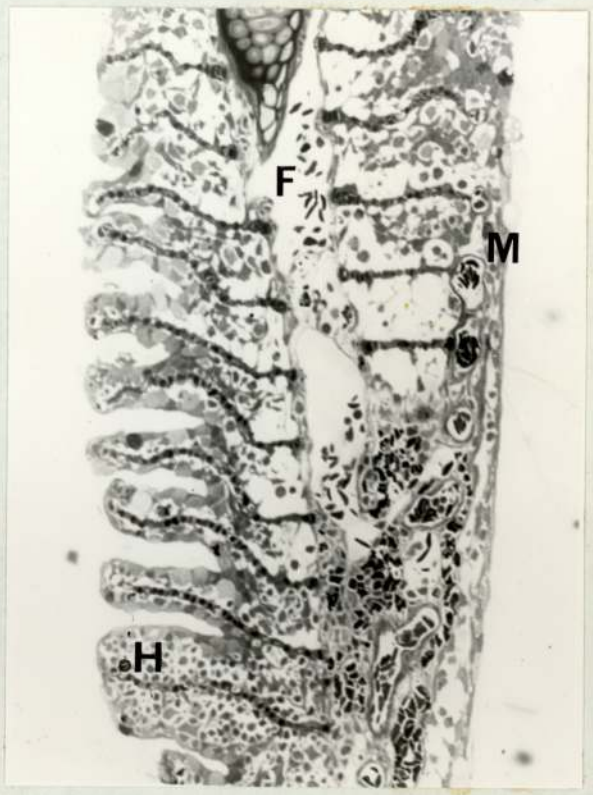


Fig.2.4. Gill section from SLS-exposed fish at opercular immobilisation. Nearly all lamella blood spaces are occluded and extensive detachment of filament and lamellar epithelium has caused the formation of large sub-epithelial lymph spaces (x 200).

Fig.2.5. This oblique section shows several features typical of the later stages of poisoning : enlarged lamellar marginal channels (M); erythrocytes in the filamental sinus (F); blockage of lamellar blood spaces, epithelial detachment and haematoma (H). (See also Fig.2.10) (x 200).



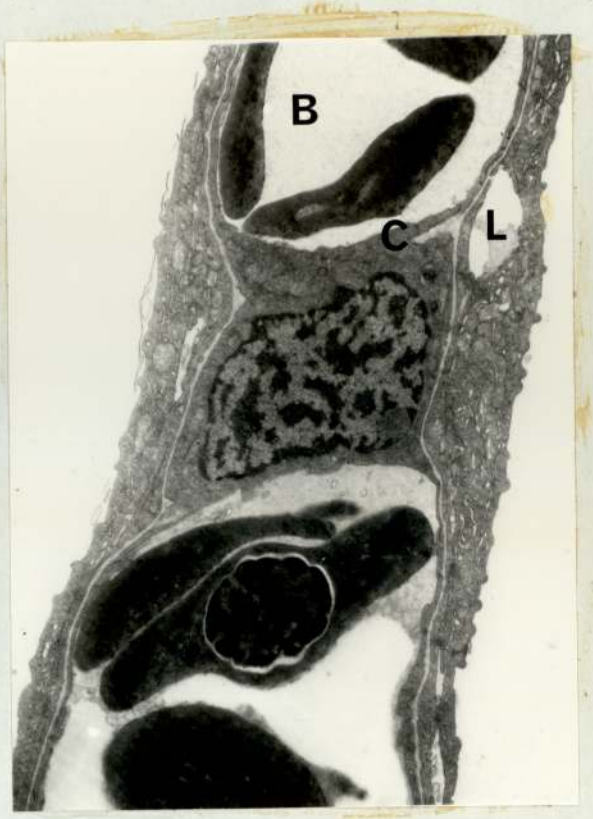


Fig.2.6. Electron micrograph of control gill secondary lamella in transverse section. The blood spaces (B) are lined by the pillar cell flanges and contain erythrocytes. Part of a pillar cell column (C) is visible. The epithelium is closely apposed to the underlying tissue except for a small lymph space (L) between the cell layers (x 7500).

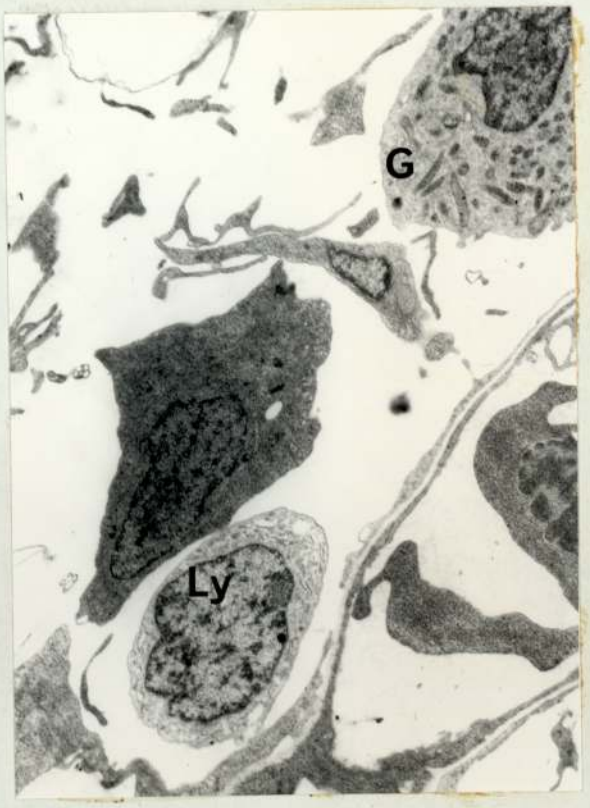


Fig.2.7. Transverse section through a secondary lamella of an SLS-exposed fish at 60% LT50. The subepithelial space contains granulocytes (G), lymphocytes (Ly) and loose epithelial cells (x 7500).

Fig. 2.8. Collapsed pillar cell system in a secondary lamella of a fish exposed to SLS until opercular immobilisation, showing an occluded blood space (B) and its adjacent pillar cell (P). The pillar cell column (C) is clearly continuous with the basement membrane. S, subepithelial lymph space (x 22500).

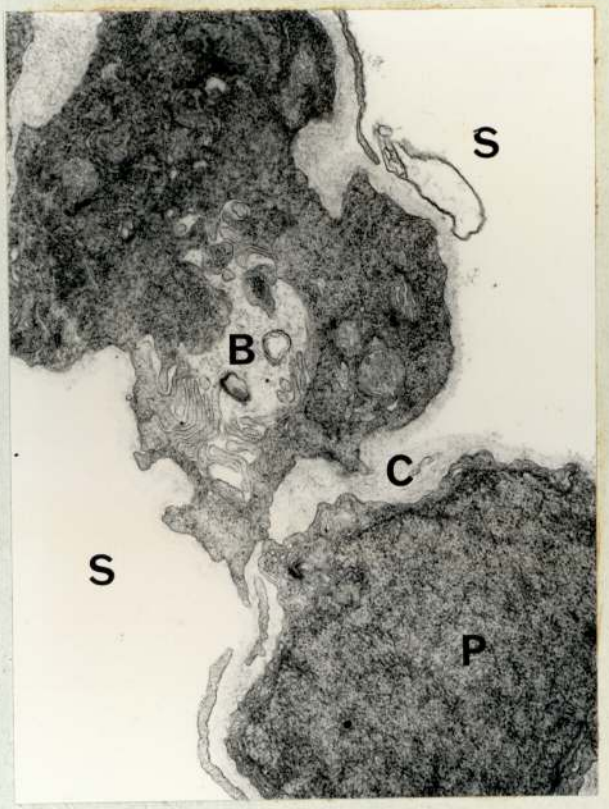


Fig. 2.9. Dead epithelial cell in the process of detachment from its healthier (darker-staining) counterparts. Subepithelial spaces (S) remain separated from the water (W) : desmosomal junctions (arrowed) between viable cells remain intact (x 6500).

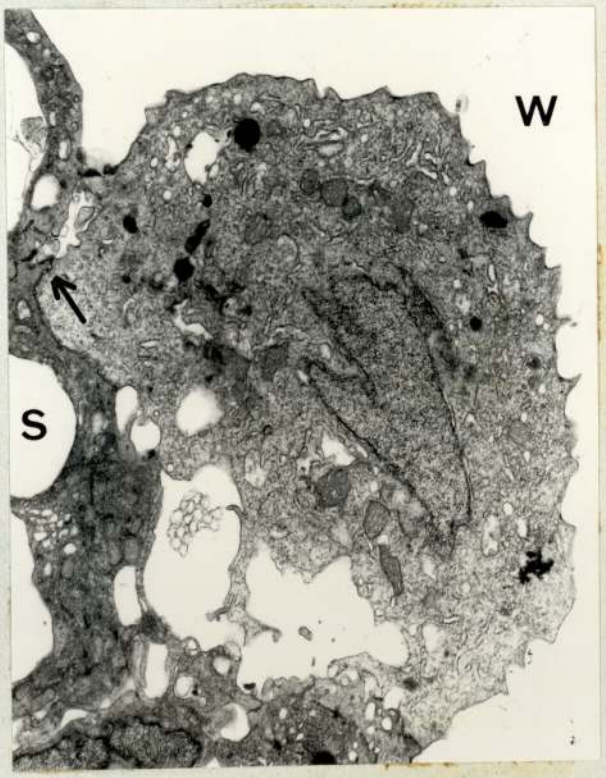


Fig.2.10. Light micrograph showing haematomas in three adjacent secondary lamellae of an overturned fish. The erythrocytes are confined within the unbroken epithelium. The pyknotic nucleus of a dying cell (arrowed) is clearly visible (x 550).

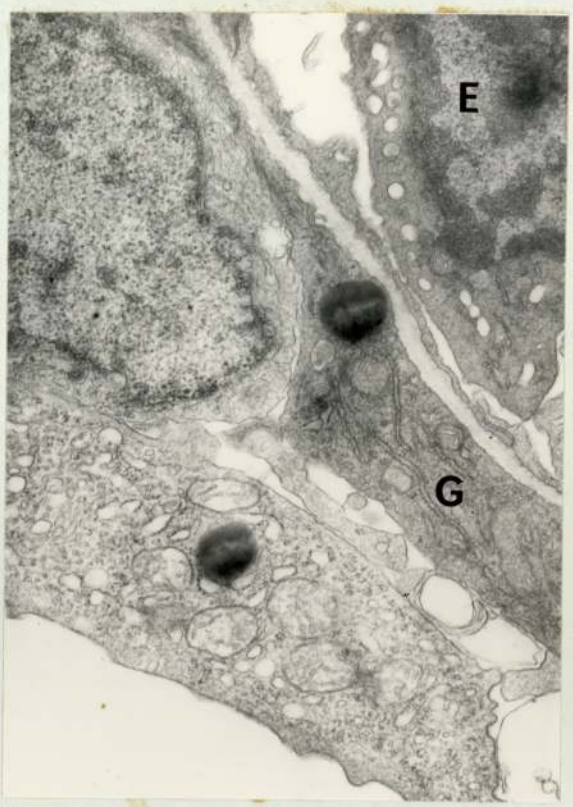


Fig.2.11. Early manifestations of SLS-induced cellular death are the formation of lysosomes and granular endoplasmic reticulum (G). The lower lysosome has formed in a chloride cell, which is about to be sloughed. Note that this cell has not lifted in the manner characteristic of zinc poisoning. Circulating eosinophilic leucocytes (E) are plentiful from 60% LT50 onwards (x 22500).

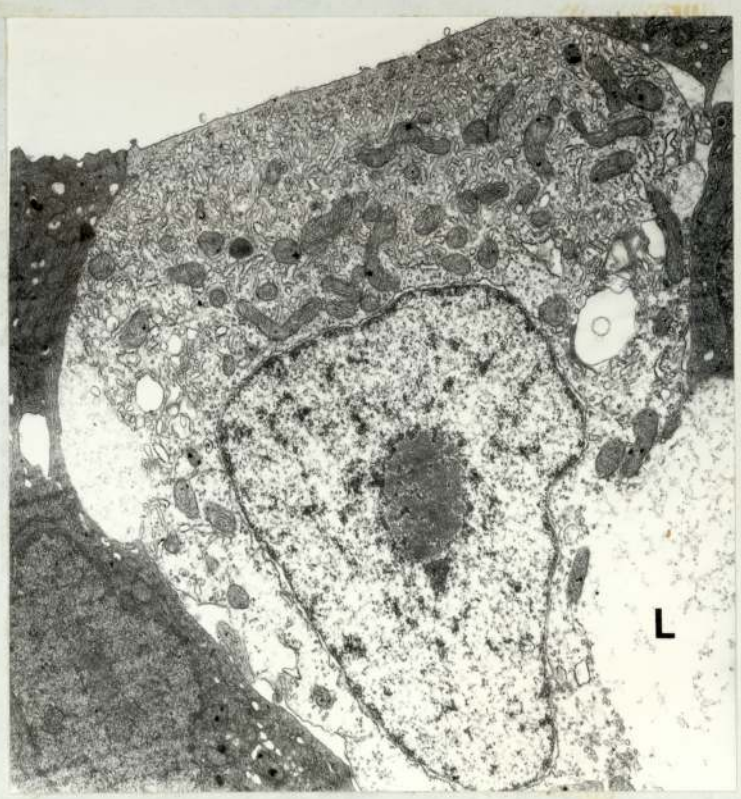


Fig.2.12. Chloride cell from a fish exposed to SLS for 30% LT50. The faint staining and nuclear pyknosis characteristic of dead and dying cells was also detectable by light microscopy (Fig.10). Note the loss of microvilli from the affected cell, cytoplasmic disintegration in the basal region and newly-formed lymph space (L) (x 11000).

CHAPTER 3. Comparative study of the effects of several lethal concentrations of SLS on the gills of trout.

3.1 INTRODUCTION

Concentrations of pollutants are regarded as acutely toxic to fish if they cause death within a period ranging from a few minutes to several days (Sprague, 1969). The effects of acutely toxic concentrations of poisons have been widely studied (Chapter 2), based on exposure times varying from less than one hour (Lindahl & Hell, 1970) to 24 h (Cairns & Scheier, 1962) or even 48 h (Brown et al., 1968). Few investigators have studied the effects of more than one concentration of poison, and there has been no systematic study of the effects of concentration on the pattern of gill damage for any poison. In this study, gill damage caused by ten different concentrations of the anionic detergent SLS, lethal over periods ranging from 6 min to 45 h has been investigated in the brown trout (Salmo trutta L.)

3.2 MATERIALS AND METHODS

Salmo trutta, mean weight 1.3g (s= ± 0.4g), mean fork length 52mm (s= ± 4mm), were obtained from a commercial hatchery and acclimated to Birmingham tap water (hardness 25 mg/l as $CaCO_3$) at 15°C. for 7 days. Fish were fed daily on pelleted food, except during and on the day preceding the experiments.

All exposures were carried out at 15 ± 1°C., in batches of eight to ten fish in glass aquaria containing 10 litres of test solution, gently aerated. Detergent concentrations were monitored by the methylene blue method of Degens et al. (1953). At approximately 8 h intervals, 80% of the test solution was siphoned out and replaced with fresh solution. Concentrations were maintained within ± 10% of their nominal value.

The fish were observed continuously for the first few hours of the experiment, and thereafter at frequent intervals. Mortalities in each tank were recorded and cumulative percentage mortality on a probit scale was plotted for each concentration against elapsed time on a logarithmic

scale (Bliss, 1937). Median survival times and their 95% confidence limits were estimated by the method of Litchfield (1949).

Gill tissue was collected for examination from fish observed in dying. Tissue was collected, prepared and examined with light and electron microscopes by the methods described in Chapter 2.

3.3 RESULTS

Median survival times and their 95% confidence limits are shown in Table 3.1.

A. Normal gill structure

The structure of S. trutta gill does not differ in any significant respect from that of S. gairdneri as described in Chapter 2. Light and electron micrographs of control S. trutta gill are shown in Figs. 3.1 and 3.6.

B. Toxic effects of SLS

At the two lowest concentrations tested, 18 and 32 mg/l, the condition of the gills at death was as shown in Figs. 3.2, 3.7, 3.8, 3.9 and 3.10. The epithelial cells were swollen so as to occlude almost completely the interlamellar spaces through which water normally flows (Figs. 3.2, 3.7, 3.8). Lamellar blood spaces were open and contained apparently normal blood cells, although the pillar cells often appeared slightly smaller than normal. There was extensive intercellular vacuolation, and some detachment of the epithelium from both filaments and secondary lamellae. However, the extent of this oedema was very slight by comparison with the situation described in Chapter 2 in gills exposed to 100 mg/l SLS, and there was no indication of the collapse of the pillar cell system. Leucocytic invasion was present, mainly in the filamental sinus but to a slight extent in the sub-epithelial spaces also (Figs. 3.7, 3.8). Lysosome formation and nuclear pyknosis were common in epithelial cells, and sloughing of dead and dying cells was observed (Figs. 3.8, 3.9, 3.10). Lysosomes were especially common in filament cartilage cells.

At 56 and 100 mg/l, the action of SLS at the cellular level was the same as at the lower concentrations tested. Cellular death, indicated by nuclear pyknosis, faint cytoplasmic staining and lysosome formation was occurring more rapidly, however, as was shown by the quantity of sloughed cellular debris. At the tissue level, it was apparent that some features of the damage were more exaggerated forms of that caused by the lower concentrations. The formation of subepithelial lymph spaces was more pronounced (Fig. 3.3), as was the extent of leucocytic invasion of these spaces. In addition, the total occlusion of lamellar blood spaces was common (Fig. 3.11) and some haematomas were observed (Figs. 3.3, 3.11).

At concentrations of 120 mg/l and above, the action of SLS at both the cellular and tissue levels was markedly different. The damage was similar at all concentrations between 120 and 1000 mg/l inclusive, although at concentrations up to and including 180 mg/l action of the second type occurred mainly at the lamellar tips (Fig. 3.4). This action consisted of rapid cell lysis throughout the epithelium, which became completely disrupted (Figs. 3.5, 3.12, 3.13). Prior to lysis, cells became deeply stained with both methylene blue and electron-dense stains, and intracellular detail was obscured (Fig. 3.13). Pillar cells in this condition appeared much reduced in size, though in the lamellar distal regions they were usually of normal size and killed by detergent action (Figs. 3.12, 3.13). It appeared that this type of damage progressed from the distal to the proximal regions of the lamellae, since the distal lamellar regions were always more severely damaged and concentrations of 120 to 180 mg/l in some cases affected only the lamellar tips in this way.

At all concentrations of 120 mg/l and above, circulating erythrocytes in the branchial and filament arteries were severely deformed, indicating incipient haemolysis (Fig. 3.14). Electron microscopically, these cells showed an unusual pattern of staining (Fig. 3.15).

3.4 DISCUSSION

Although the action of acutely-lethal concentrations of poisons on fish gills has been widely studied (Chapter 2), few investigations have taken account of the time over which the poisons act. This study has revealed that the pattern of gill damage varies with the concentration even among concentrations of toxicant which may all be regarded as acutely lethal. It is therefore desirable, for the sake of comparability of results, that in studies of toxicant-induced gill damage the median survival times of fish exposed to the concentrations tested are determined and stated.

The pathological effect of SLS in concentrations up to about 100 mg/l is an acute inflammatory response (Anderson, 1966), characterised by leucocytic invssion and oedema of the gill tissue. With increasing concentration, the severity of the inflammation increases. Epithelial lifting caused by the loss of function of epithelial cells, according to the mechanism suggested in Chapter 2, may be a contributory factor to the extent of oedema. However, the sub-epithelial spaces appear to maintain normal osmotic conditions (Chapter 2), indicating that the fluid they contain is mainly of internal origin and is not formed by an influx of water. Since the pattern of damage at the cellular level is similar in all concentrations of 100 mg/l and below, it appears that the pattern of gill damage at the tissue level depends upon the rate at which epithelial cells are killed by toxicant action.

At very high acutely-lethal concentrations, there is a definite change in the effect of SLS at the cellular level, characterised by the rapid lysis of cells, and penetration by the detergent into the blood spaces and pillar cells. The effect at the cellular level is so rapid and widespread that no reorganisation of the epithelium such as occurs at lower concentrations can take place. Overall lamellar structure is therefore retained, the gill tissue being dead and unresponsive to either locally or centrally co-ordinated control mechanisms. As with lower

concentrations, it appears that the pathology of gill damage at the tissue level is governed by the rate and nature of the toxicant action at the cellular level.

The nature of detergent action at the cellular level has been widely studied using bacterial cells and protoplasts, various other micro-organisms and erythrocytes. A survey of the biomedical literature offers some striking parallels between detergent action in isolated cells of this type and in the present study (Salton, 1957, 1968; James, 1964; Hugo, 1967). It is generally agreed that there are two possible mechanisms whereby detergents can cause cell death. The first mechanism was suggested by Hotchkiss (1948), who demonstrated the release of nitrogen- and phosphorus-containing compounds from bacterial cells exposed to detergents before any detectable lysis took place. Hotchkiss' interpretation of these results, since amply confirmed by other investigators (Salton, 1957, 1968; Hugo, 1967), was that detergents disorganise the cell's permeability barrier, resulting in leakage of metabolites and possibly enzymes and co-enzymes. The cell becomes non-viable and autolysis occurs, i.e. lysis occurs by the cell's own enzymic processes and not by the direct action of the detergent. Further investigations (discussed by James, 1964), demonstrated that the lipid component of the membrane was the probable primary site of detergent action. The observed sequence of events in cell death induced in gills by concentrations of SLS between 18 and 100 mg/l inclusive involves nuclear pyknosis, the formation of lysosomes and eventual dissolution of cell contents, as described here and in more detail in Chapter 2. This sequence of events is compatible with the mechanism described above by which detergents affect isolated cells in vitro, and it is suggested that this same mechanism is responsible for the action of detergents on gills at concentrations below about 120 mg/l.

The second mechanism of detergent bactericidal action, lysis by direct action of the detergent on the protein component of membranes and

cell walls, is similarly compatible with the observed nature of the damage caused to gill cells by high concentrations of SLS. Detergents are known to be protein denaturants (Putnam, 1948; Glassman, 1948), and it is widely accepted that this mechanism is the basis of bactericidal action in certain cases (Salton, 1957, 1968; James, 1964).

The possibility of a concentration-dependent change in the mechanism of action of detergents on bacteria does not appear to have been investigated, and direct comparison between gill cells and bacterial cells is not possible because of the difference in cell structure. The effects of concentration on the interaction of detergents and proteins *in vitro* have been widely studied (Putnam, 1948; Glassman, 1948), and the occurrence of concentration-dependent changes in the interaction between detergents and proteins is well established. Depending on the relative concentration of detergent and protein in solution, detergents may bind reversibly or irreversibly to proteins, and may produce reversible or irreversible denaturation or precipitation. Unfortunately it is not possible to compare directly the results of *in vitro* studies with the effects of detergents on gills, but it is interesting to note that pH also has a marked effect on detergent/protein interactions *in vitro*. In this context it should not be overlooked that the pH at the gill surface is probably more acid than that of the water generally (Lloyd & Herbert, 1960). Further investigation of the action of detergents at the cellular level may profitably be carried out using cultured gill cells *in vitro*.

Although the action of SLS at the cellular level and its consequent effects on gill structure may thus be explained, it cannot be assumed that its toxic action in the whole animal is confined to the gill surface. Haemolysis of cells in the branchial artery (Figs. 3.14, 3.15) demonstrates clearly that SLS enters fish exposed to very high concentrations. While it is reasonable to expect that death of fish exposed to any concentration of SLS tested may be due to hypoxia as a consequence of gill damage (Skidmore, 1970), such a conclusion would be premature in the

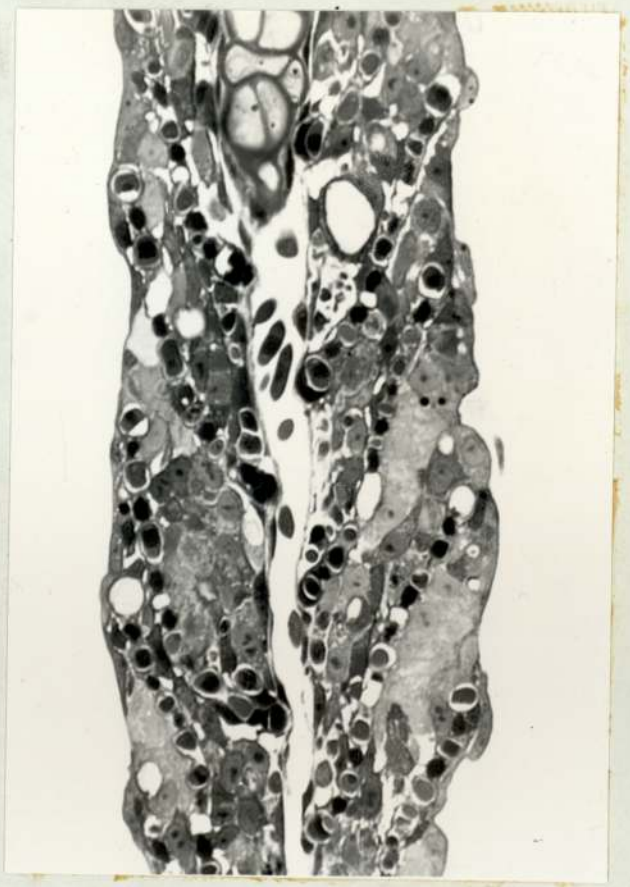
absence of physiological studies to determine the extent to which detergents enter fish and exert toxic action internally, and on functions other then respiration.

TABLE 3.1 Median survival times and their 95% confidence limits for *S. trutta* exposed to SLS.

Concentration (mg/l)	Median Survival time (h)	Confidence limits
1000	0.07	0.06 - 0.09
560	0.07	0.06 - 0.08
320	0.08	0.07 - 0.09
180	0.15	0.12 - 0.19
150	0.26	0.23 - 0.29
120	0.86	0.74 - 1.0
100	2.15	1.7 - 2.6
56	6.5	5.6 - 7.5
32	32	25 - 42
18	45	37 - 55

Fig.3.1. Light micrograph showing gill of control fish (x 500).

Fig.3.2. Light micrograph of gill from a fish killed by exposure to 18 mg/l SLS. The interlamellar water spaces are occluded by the swollen epithelial cells (x 500).



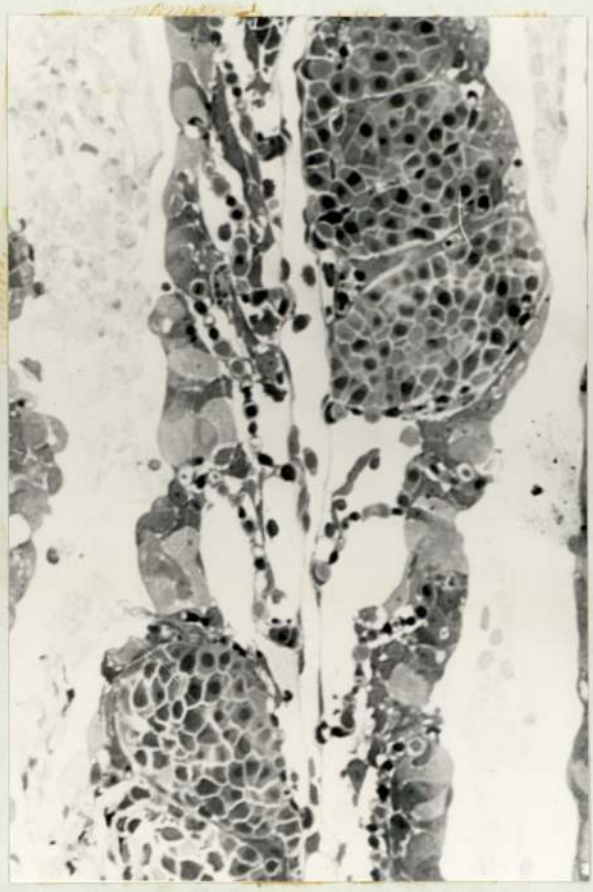


Fig.3.3. Light micrograph of gill from fish killed by 56 mg/l SLS. Note the large subepithelial spaces and collapsed pillar cell system, haematomas and large quantity of sloughed cellular debris (x 400).

Fig.3.4. Light micrograph of gill from fish killed by 120 mg/l SLS. Note the appearance of the second type of action at the lamellar tips (x 500).

Fig.3.5. Light micrograph of gill from fish killed by 560 mg/l SLS. The epithelium is completely disrupted owing to the lysis of its cells. Blood spaces are open and contain lysed blood cells. Note that cells not yet lysed are very deeply stained. (x 500).

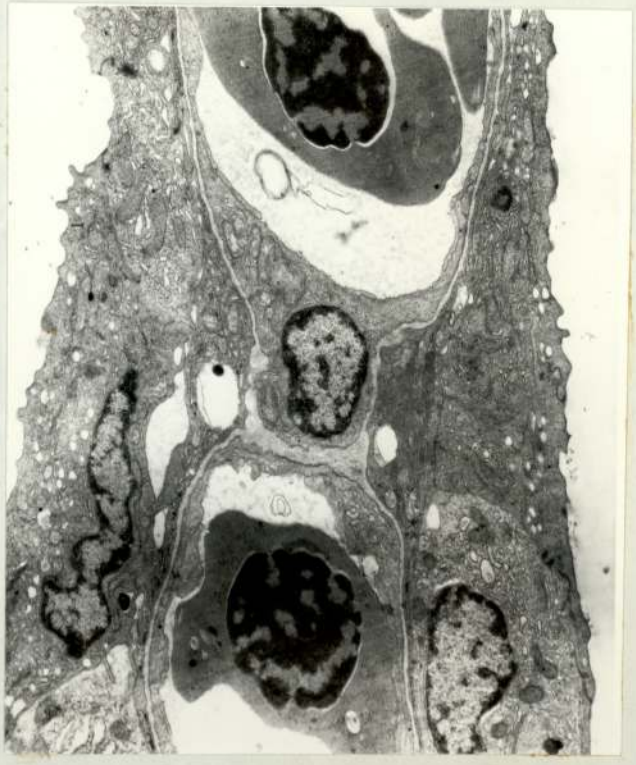


Fig.3.6. Electron micrograph of control gill lamella (x 7500).

Fig. 3.7. Electron micrograph of gill from fish killed by 18 mg/l SLS, showing two adjacent secondary lamellae. The interlamellar spaces are occluded by the swollen epithelium. Note the slight oedema, leucocytic invasion (L), open blood spaces and large water - blood diffusion distance (x 4500).

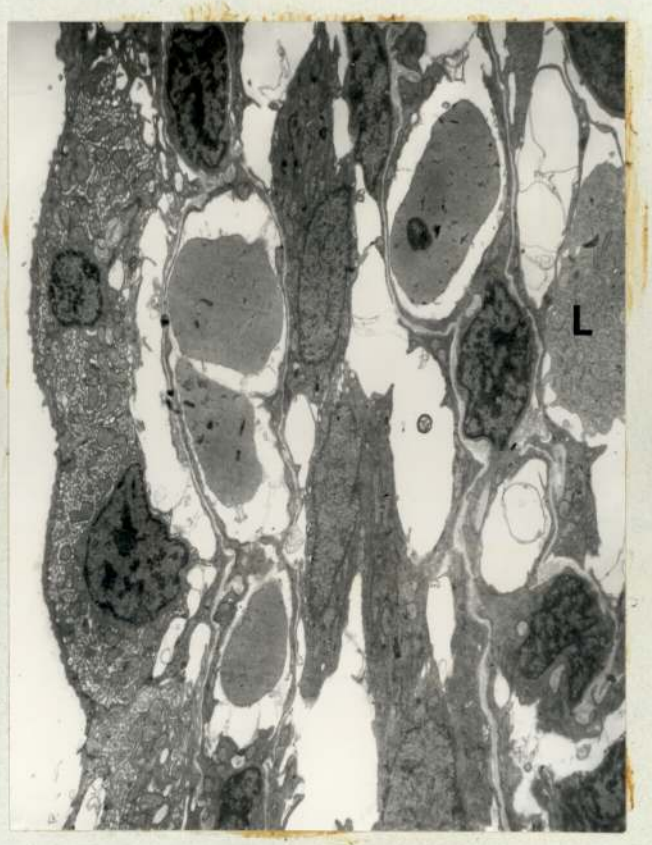


Fig. 3.8. Electron micrograph of gill from fish killed by 18 mg/l SLS, showing leucocytic invasion (L), nuclear pyknosis (N) and the greatly enlarged water - blood diffusion distance. M, lamellar marginal channel (x 7500).

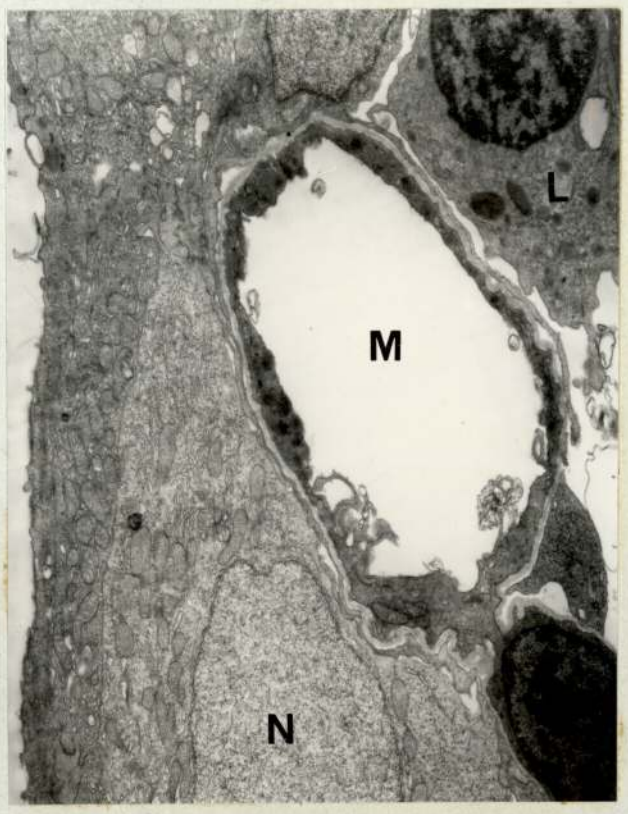


Fig.3.9. Sloughing of a dead epithelial cell from the gill of a fish killed by 32 mg/l SLS. (x 9500).

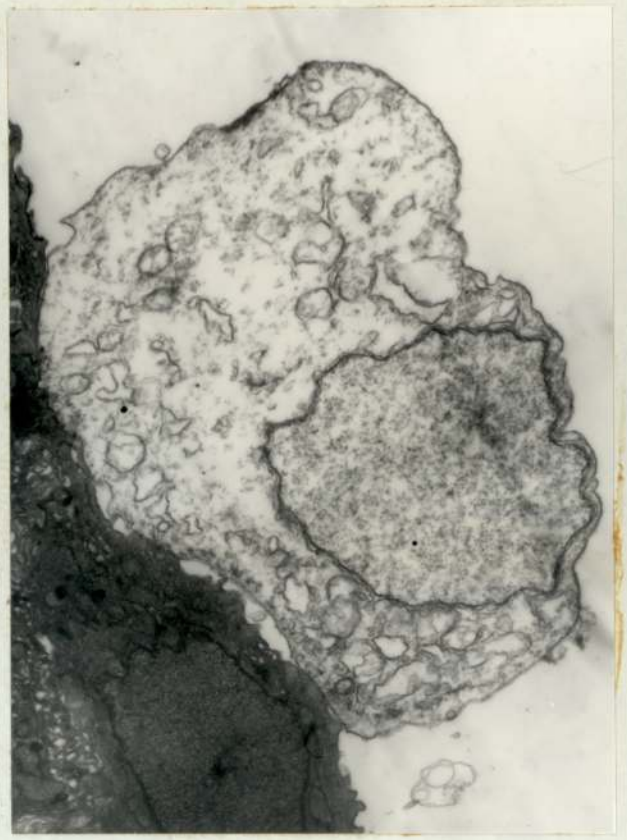
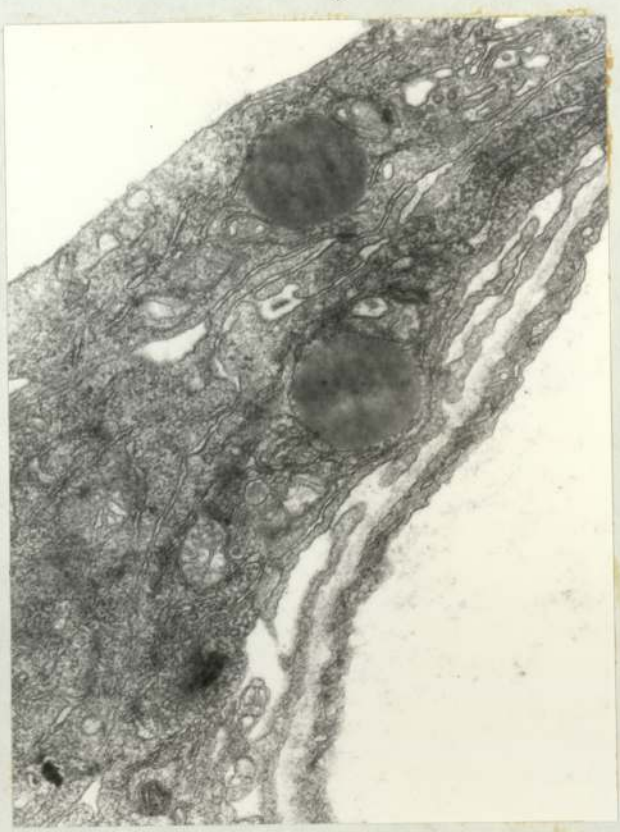


Fig.3.10. Lysosome formation in the gill epithelial cells of a fish killed by 32 mg/l SLS (x 30000).



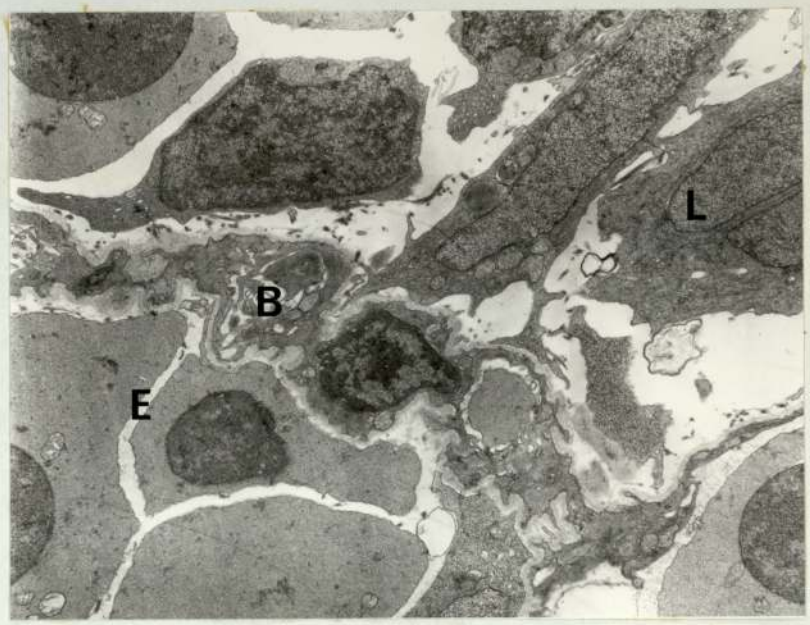


Fig.3.11. Electron micrograph showing the collapsed pillar cell system in the gill of a fish killed by 56 mg/l SLS. Note occlusion of the lamellar blood spaces (B), polymorphonuclear leucocyte (L), and haematoma as shown by erythrocytes (E) outside the pillar cell system.

Fig.3.12. Lamellar tip from fish killed by 180 mg/l SLS. Note disrupted epithelium with complete lysis of cells, open blood spaces with lysed erythrocytes, and dead pillar cells (x 4800).

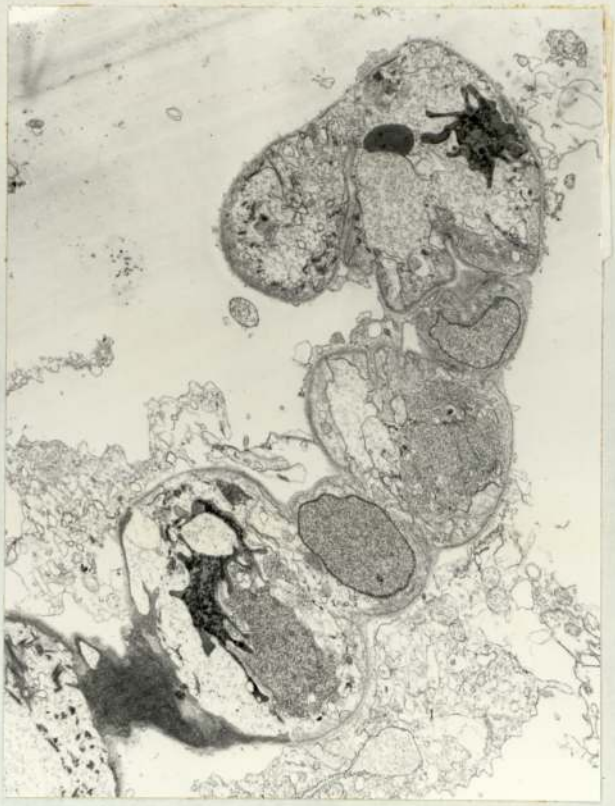


Fig.3.13. Lamellar base from gill of fish killed by 180 mg/l SLS. Note the very reduced size of the pillar cells, and the dense staining and loss of cytoplasmic detail in cells prior to lysis (x 4500).

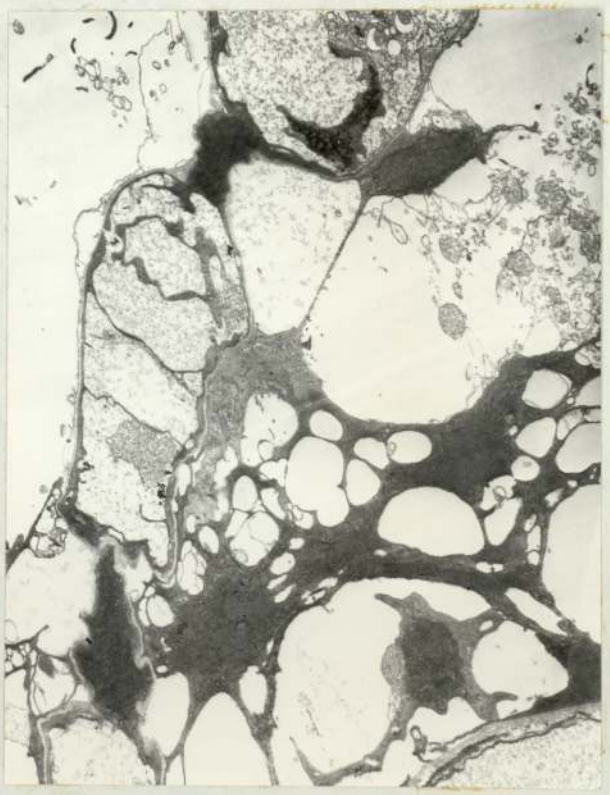


Fig.3.14. Deformed blood cells in the branchial artery of a fish exposed to 120 mg/l SLS (x 500).

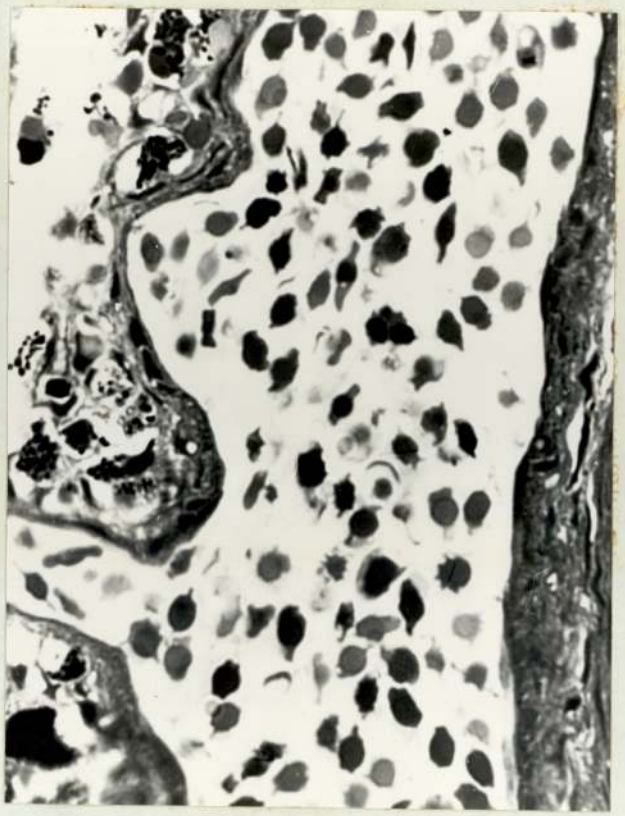
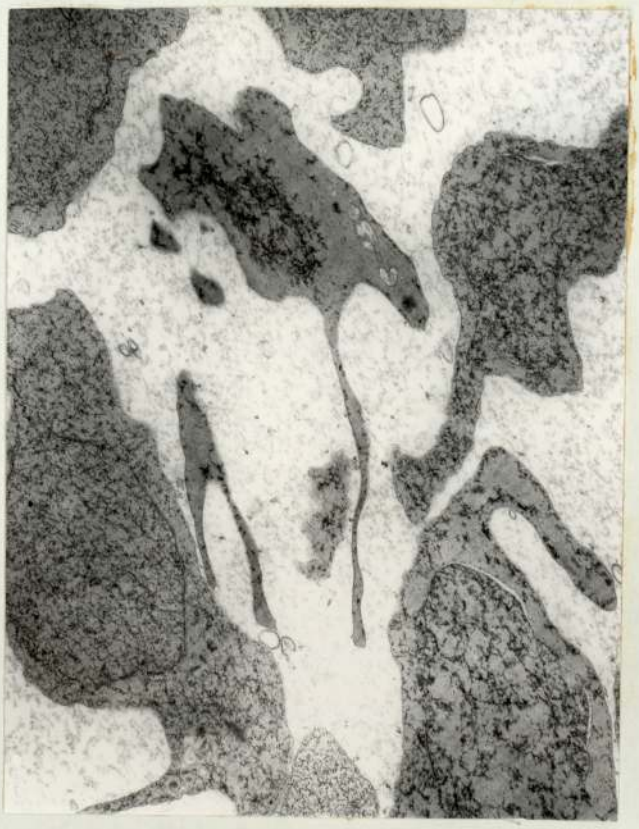


Fig.3.15. Electron micrograph of erythrocytes in the branchial artery of a fish killed by 150 mg/l SLS. The deformity of the cells indicates incipient haemolysis, but the significance of the abnormal staining of the cells is not clear (x 7500).



CHAPTER 4. Mortality patterns of trout exposed to SLS in relation to concentration and mechanisms of toxic action.

4.1 INTRODUCTION

In toxicity testing it is normal practice to determine the survival times of fish exposed in groups of about ten to a range of concentrations of poison. For each concentration, a graph is plotted of cumulative percentage mortality (usually transformed to a probit scale) against time (usually transformed to a logarithmic scale). From these so-called 'probit lines', a value of median survival time (LT50) is determined for each concentration, and LT50 is plotted against concentration on a log-log scale to give the line relating median survival time to concentration of poison, often called the toxicity curve. The standard methods and their variations have been described in some detail by Sprague (1969).

Occasionally irregularities in the form of inflexions in probit lines or toxicity curves are detected. One possible cause of such irregularities is that mortality is occurring as a result of more than one mechanism of toxic action. Tyler (1965) interpreted irregularities in the probit responses of minnows (Chrosomus spp.) exposed to high temperatures as indicating that three separate mechanisms of death were operative. Changes in the slope of probit lines may also be thus interpreted(Litchfield & Wilcoxon, 1949; Sprague, 1969; Burton et al., 1972). Herbert & Downing (1955) found an inflexion in the toxicity curve of trout exposed to potassium cyanide and concluded that "a biological difference exists between the two regions into which we have divided the concentration/survival-time relationship." Sprague (1969) cites several examples where irregularities of this sort have been explained in terms of multiple mechanisms of toxic action.

It was shown in Chapter 3 that when trout are poisoned by a range of concentrations of the anionic detergent SLS, a change in the mode of action of the detergent occurs at a concentration around 120 mg/l as shown by its pathological effects on the gills. Toxicity tests were

therefore conducted in order to study the resulting probit lines and toxicity curves in relation to this concentration-dependent change in the toxic action of the detergent. The object of these experiments was to determine whether the observed change in the mode of toxic action of the poison was reflected in the probit lines and toxicity curves, and thus to investigate the validity of interpreting the properties of these curves in terms of multiple mechanisms of toxic action.

Data relating to Salmo trutta presented in this chapter are those obtained during the experiment described in Chapter 3. Throughout this chapter, the action of SLS which results in the autolysis of the gill cells is termed the first type of toxic action. The action of SLS which results in rapid direct lysis of the cells is termed the second type of toxic action. The two actions have been described fully in Chapters 2 and 3.

4.2 MATERIALS AND METHODS

Two species of trout of different ages were used, Salmo trutta (age approximately 150 days, mean weight 1.3g, s = 0.4g, mean fork length 5.2 cm, s = 0.4 cm), and Salmo gairdneri (age approximately 1 year, mean weight 25.6g, s = 9.8g, mean fork length 12.6 cm, s = 1.9 cm). Fish were obtained from a hatchery and acclimated to Birmingham tap water (total hardness 25 mg/l as $CaCO_3$) at 15 $\pm$ 1°C for at least 7 days prior to the experiments. Feeding was by pelleted food daily except during and on the day preceding experiments. All experiments were carried out in Birmingham tap water at 15 $\pm$ 1°C.

Salmo trutta were exposed in batches of eight to ten fish in glass aquaria containing 10 litres of gently aerated test solution. Salmo gairdneri were exposed in groups of ten in steel-framed glass aquaria containing 40 litres of gently aerated test solution. At approximately 8 h intervals, 80 per cent of the test solution was removed and replaced with fresh solution. Detergent concentrations were monitored by the methylene blue method (Degens et al., 1953), and were maintained within $\pm$

10 per cent of their nominal value. It was found impossible to maintain these concentration limits in the large aquaria for more than 36 h, so the experiment with the rainbow trout was terminated at this time. Fish were exposed to a range of concentrations between 18 and 1000 mg/l SLS (Tables 4.1 and 4.2). Exposures of S. gairdneri to concentrations of 120 and 150 mg/l were carried out in duplicate.

Fish were observed continuously for the first few hours of the experiments, and thereafter at frequent intervals. Mortalities in each tank were recorded and cumulative percentage mortality was plotted on a probit scale against elapsed time on a logarithmic scale (Bliss, 1937). Median survival times and their 95 per cent confidence limits were estimated by the graphical method of Litchfield (1949) and plotted against concentration of SLS on logarithmic scales. For each species, each probit line was tested for parallelism against every other line, also by the method of Litchfield (1949).

Gill tissue was collected from dying fish, and prepared for microscopical examination by the methods described in Chapter 2.

4.3 RESULTS

A. Effects of SLS on gills.

The condition of the gills at death was described in detail for S. trutta in Chapter 3. Gills of S. gairdneri were studied less extensively, but in general showed similar patterns of damage. As with S. trutta, the rapid lytic action of SLS on the gill cells and the associated haemolysis occurred at concentrations of SLS of 120 mg/l and above. Gills of the most resistant and least resistant individual S. gairdneri exposed to concentrations of 120 and 150 mg/l SLS were examined separately, but did not differ in their pathological condition.

B. Probit lines.

Some examples of probit lines are shown in Figs. 4.1 and 4.2. Except for the examples shown, no irregularities in probit lines were detected. At 120 mg/l the first exposure of a batch of ten fish

resulted in a split probit line (Fig. 4.1B), and it is the data from this line which is included in Tables 4.2 and 4.5. A second exposure of ten fish to 120 mg/l SLS did not give a split probit line (Fig. 4.1A).

Median survival times and their 95 per cent confidence limits for the two species of fish are shown in Tables 4.1 and 4.2. Values for the slope function, S, of the probit lines (Litchfield, 1949) are also shown. Data relating to the duplicate exposures of S. gairdneri to concentrations of 120 and 150 mg/l SLS are shown in Table 4.3, and are very close in value to the results of the first exposures.

Results of the tests for parallelism of the probit lines for each species are shown in Tables 4.4 and 4.5. For S. trutta, all lines are parallel within the limits of experimental error, except that the line for 150 mg/l is not parallel to the lines for 180, 100 or 32 mg/l. For S. gairdneri, all lines are parallel within the limits of experimental error except that the line for 150 mg/l is not parallel to the lines for 1000, 560, 320, 120, 100 or 42 mg/l.

C. Toxicity curves.

Median survival times and their 95 per cent confidence limits plotted against concentrations of SLS on logarithmic scales are shown in Figs. 4.4 and 4.5. Both sets of points are reasonably well fitted by a single curve which approaches an asymptote on either axis.

4.4 DISCUSSION

Irregularities in the curves expressing the relationship between mortality, survival time and concentration of poison are frequently interpreted in the absence of other information as indicating changes in the nature of toxic action. A concentration-dependent change in the toxic effects of SLS at the cellular level is observable by light and electron microscopical examination of the gills of trout exposed to the detergent. The purpose of these experiments was to determine whether this change is reflected in the toxicity relationships of trout exposed to SLS. Mortality curves for fish exposed to a concentration of

ERRATUM

The sentence beginning on line 26 of page 51 should read:

"Over a narrow range of concentration and survival time, the concentration of SLS is sufficiently high to exert both types of toxic action, while the response time is sufficiently long to allow the first type of toxic action to contribute to mortality."

150 mg/l SLS are significantly different in slope from mortality curves for other concentrations. The occurrence of a change in the toxic effects of SLS on the gills at a concentration around 120 mg/l furnishes strong circumstantial evidence that the deviation from parallelism of the 150 mg/l lines is associated with a change in the nature of the toxic action of SLS on the fish.

The straightness of a probit line indicates that mortality times of individual fish are normally distributed, and the slope function of the line represents the variance of this distribution. The test for parallelism is, in effect, a test of the significance of the difference in variances between two normal distributions. Where two modes of toxic action occur, there is the possibility that the probit lines will be divided into two groups, each having a characteristic variance (slope function) differing in value from that of the other group. This does not appear to be the case here, where only the 150 mg/l line deviates from parallelism with respect to the other lines. A possible interpretation of this result is as follows. Within all the groups of fish tested, the individual trout may be equally variable in their responses to either of the two toxic actions, and it is only over the concentration range where both actions occur simultaneously that a change in variability of resistance is evident. At the lower concentrations, the response time of the fish is comparatively slow, while the detergent concentration is insufficient to exert toxic action of the second type. At high concentrations the response time is very short, and although toxic action of both types may occur, the fish dies before the first type of action has time to manifest itself. Over a narrow range of concentration and survival time, the concentration of SLS is sufficiently long to allow the first type of toxic action to contribute to mortality. Under these circumstances, if the fish which are most susceptible to the first type of toxic action are also the most susceptible to the second type of action, the effect will be to displace the bottom end of the cumulative

mortality curve further to the left than the top end, resulting in an increase in slope (variability) and hance the deviation from parallelism.

Herbert & Downing (1955) found that the toxicity curve for Salmo gairdneri exposed to potassium cyanide was divided into two distinct regions, which they interpreted as indicating the occurrence of two different toxic actions. Since cyanide poisoning is rapidly reversible, they were able to test the comparative resistance of individual fish to the two types of toxic action, using overturn of the fish as the end point. They found that the fish which were most resistant to one toxic action did tend to be the most resistant to the second action also. This finding supports the above hypothesis, but this hypothesis is not applicable in the case of the S. trutta experiments with SLS. Here, although the 150 mg/l probit line did show deviations from parallelism its slope function was smaller, not greater, than that of the other lines. No adequate explanation of this finding can be offered; perhaps resistance to one toxic action does not necessarily confer resistance to another, depending on the size and species of the fish.

Split probit lines may be explained as follows. If the concentration of poison and the variability in resistance among the individual fish within a sample are such that the most resistant fish are not affected at all by the second toxic action, an inflexion in the probit line will occur delineating those fish affected by one action from those fish affected by both. A large number of exposures of groups of fish to concentrations within the range over which the two toxic actions overlap may therefore be expected to yield some examples of split probit lines. In fact, one of the two exposures of S. gairdneri to 120 mg/l resulted in a split probit line (Fig. 4.1). Duplicate exposures to 150 mg/l did not produce a split probit line in either case. Some weeks after the completion of the experiments described above, duplicate exposures of S. gairdneri to 130 mg/l SLS did produce a split probit line in one case (Fig. 4.5).

However, the gills of fish represented in the respective segments of the split probit lines were indistinguishable, rapid direct lysis of gill cells occurring in both sets of fish. In other words, the irregularities in the probit lines do not occur directly in response to the occurrence of the rapid lytic action of the SLS, which first occurs at the 120 mg/l concentration. Death of the fish represents an integrated response to a sequence of events initiated by a chemical interaction between the detergent and the living material. The effect of the detergent on the gills illustrates that the nature of the chemical interaction changes at about 120 mg/l, but the subsequent sequence of events has not been investigated. Factors likely to require study in this connection are the rates and routes of entry of the poison into the fish, the effects of the poison on organs and tissues other than the gills, and the physiological results of such effects. It is therefore unrealistic to interpret the shape of the probit lines solely in terms of the gill pathology. The change in toxic effect of SLS on the gills at the 120 mg/l concentration should be regarded as marking one extreme of a range of concentration over which the complex sequence of events usually referred to as the mode of toxic action is altered.

The absence of any striking irregularities in the toxicity curves indicates that a change in the nature of the toxic action of a poison may not be reflected in the toxicity curve even if study of the probit lines and pathological effects indicates the occurrence of such a change. It is important to realise that the confidence limits of the survival time values on these curves only indicate the variation to be expected if the experiment were immediately repeated <u>using the same fish</u>. The variation in median survival times between different samples of fish would be appreciably greater, so only large irregularities in toxicity curves are likely to be detected even if the test exposures were repated many times. Small irregularities, if they exist, would lie within the limits of experimental error. In other words, the

construction of a hyperbolic toxicity curve is probably correct within the limits of experimental error.

TABLE 4.1. Median survival times and their 95 per cent confidence limits, and slope functions of probit lines for samples of ten *Salmo trutta* exposed to sodium lauryl sulphate.

Concentration of SLS (mg/l)	Median survival time (h)	95% confidence limits (h)	Slope function
1000	0.07	0.06 - 0.09	1.30
560	0.07	0.06 - 0.08	1.19
320	0.08	0.07 - 0.09	1.22
180	0.15	0.12 - 0.19	1.45
150	0.26	0.23 - 0.29	1.15
120	0.86	0.74 - 1.00	1.20
100	2.15	1.7 - 2.6	1.38
56	6.5	5.6 - 7.5	1.23
32	32	25 - 42	1.45
18	45	37 - 55	1.35

TABLE 4.2. Median survival times and their 95 per cent confidence limits, and slope functions of probit lines for samples of ten *Salmo gairdneri* exposed to sodium lauryl sulphate.

Concentration of SLS (mg/l)	Median survival time (h)	95% confidence limits (h)	Slope function
1000	0.07	0.06 - 0.08	1.29
560	0.08	0.06 - 0.09	1.29
320	0.07	0.06 - 0.09	1.27
180	0.32	0.27 - 0.38	1.35
150	0.53	0.37 - 0.75	1.76
*120	0.98	0.81 - 1.20	1.32
100	2.5	2.2 - 2.9	1.25
75	6.4	5.2 - 7.9	1.41
56	13.5	11.2 - 16.3	1.36
42	24.5	20.9 - 28.9	1.30

*Split probit line. 6/10 reacted, $N_2 = 8$ (Litchfield, 1949).

TABLE 4.3. Mortality data from duplicate exposures of *Salmo gairdneri* to 120 mg/l and 150 mg/l SLS.

Concentration of SLS (mg/l)	Median survival time (h)	95% confidence limits (h)	Slope function
150	0.49	0.35 - 0.68	1.71
120	1.55	1.27 - 1.86	1.38

TABLE 4.4. Results of the tests for parallelism of probit lines for *Salmo trutta* exposed to SLS, according to the method of Litchfield (1949). Data from Table 4.1.

Curves compared	Slope function ratio	Result
1000/560	1.09	parallel
1000/320	1.07	parallel
1000/180	1.12	parallel
1000/150	1.13	parallel
1000/120	1.08	parallel
1000/100	1.06	parallel
1000/56	1.06	parallel
1000/32	1.12	parallel
1000/18	1.04	parallel
560/320	1.03	parallel
560/180	1.22	parallel
560/150	1.04	parallel
560/120	1.01	parallel
560/100	1.16	parallel
560/56	1.03	parallel
560/32	1.22	parallel
560/18	1.13	parallel
320/180	1.19	parallel
320/150	1.06	parallel
320/120	1.02	parallel
320/100	1.13	parallel
320/56	1.01	parallel
320/32	1.19	parallel
320/18	1.11	parallel
180/150	1.26	NOT PARALLEL
180/120	1.21	parallel

TABLE 4.4. Continued

Curves compared	Slope function ratio	Result
180/100	1.05	parallel
180/56	1.18	parallel
180/32	1.00	parallel
180/18	1.08	parallel
150/120	1.04	parallel
150/100	1.20	NOT PARALLEL
150/56	1.08	parallel
150/32	1.26	NOT PARALLEL
150/18	1.18	parallel
120/100	1.15	parallel
120/56	1.03	parallel
120/32	1.21	parallel
120/19	1.13	parallel
100/56	1.12	parallel
100/32	1.05	parallel
100/18	1.02	parallel
56/32	1.18	parallel
56/18	1.10	parallel
32/18	1.08	parallel

TABLE 4.5. Results of the tests for parallelism of the probit lines for *Salmo gairdneri* exposed to SLS, according to the method of Litchfield (1949). Data from Table 4.2.

Curves compared	Slope function ratio	Result
1000/560	1.00	parallel
1000/320	1.02	parallel
1000/180	1.05	parallel
1000/150	1.32	NOT PARALLEL
1000/120	1.02	parallel
1000/100	1.03	parallel
1000/75	1.09	parallel
1000/56	1.05	parallel
1000/42	1.01	parallel
560/320	1.01	parallel
560/180	1.05	parallel
560/150	1.38	NOT PARALLEL
560/120	1.03	parallel
560/100	1.03	parallel
560/75	1.09	parallel
560/56	1.05	parallel
560/42	1.01	parallel
320/180	1.06	parallel
320/150	1.38	NOT PARALLEL
320/120	1.04	parallel
320/100	1.02	parallel
320/75	1.11	parallel
320/56	1.07	parallel
320/42	1.02	parallel
180/150	1.30	parallel
180/120	1.02	parallel
180/100	1.08	parallel

TABLE 4.5. Continued

Curves compared	Slope function ratio	Result
180/75	1.04	parallel
180/56	1.01	parallel
180/42	1.04	parallel
150/120	1.33	NOT PARALLEL
150/100	1.41	NOT PARALLEL
150/75	1.25	parallel
150/56	1.29	parallel
150/42	1.35	NOT PARALLEL
120/100	1.06	parallel
120/75	1.07	parallel
120/56	1.03	parallel
120/42	1.02	parallel
100/75	1.13	parallel
100/56	1.09	parallel
100/42	1.04	parallel
75/56	1.04	parallel
75/42	1.08	parallel
56/42	1.05	parallel

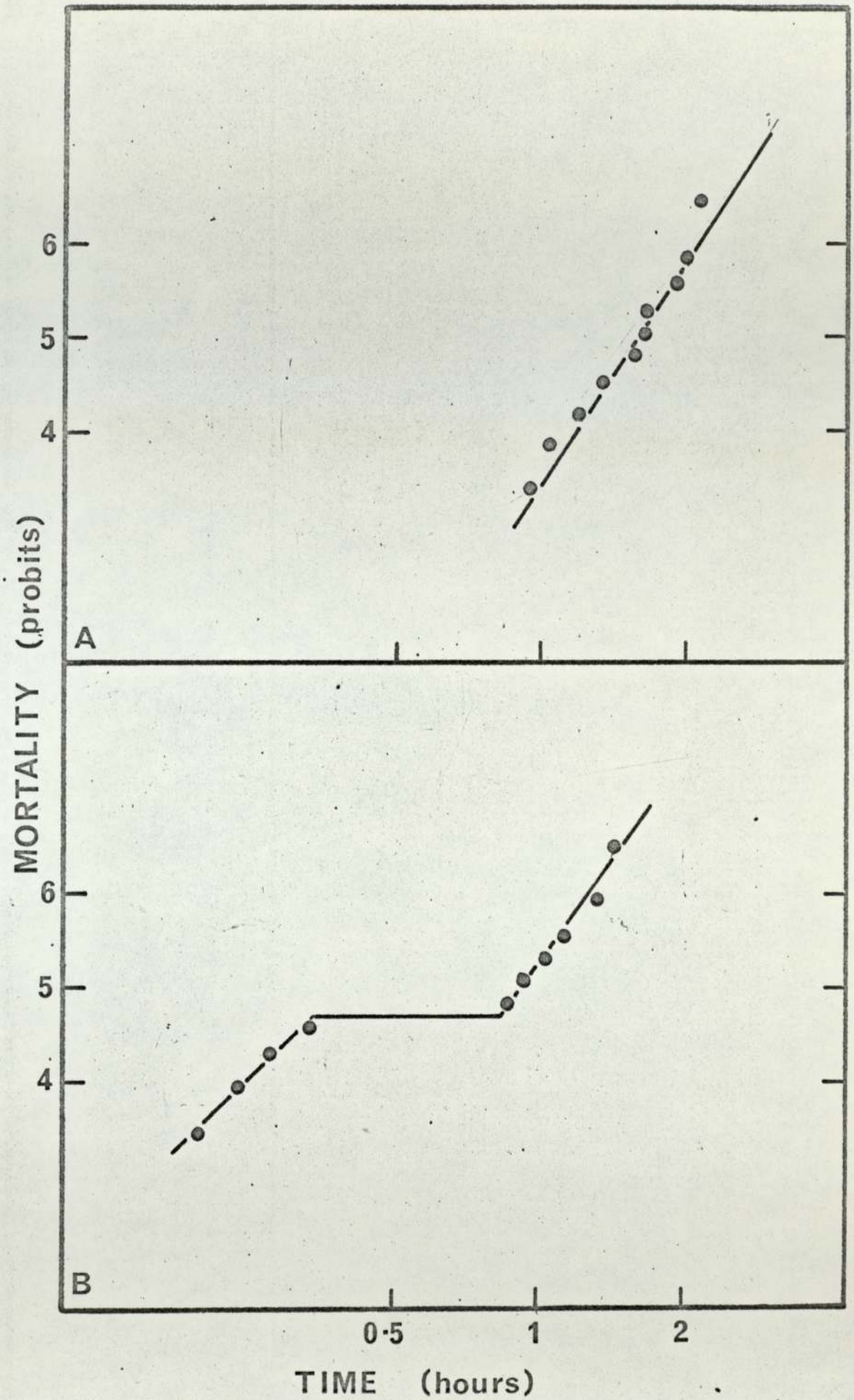


Fig. 4.1. Mortality curves for Salmo gairdneri exposed to 120 mg/l SLS

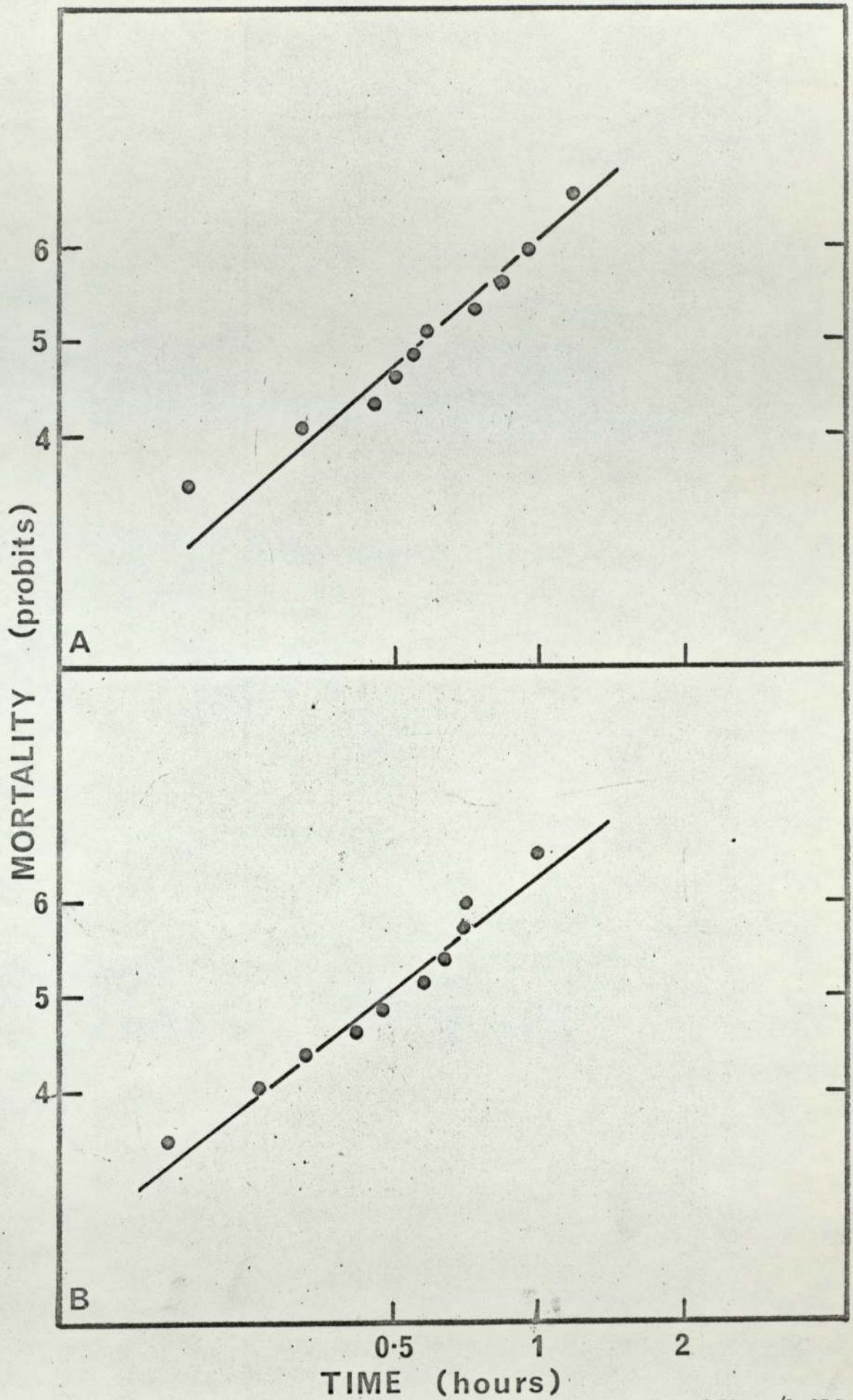


Fig. 4.2. Mortality curves for Salmo gairdneri exposed to 150 mg/l SLS

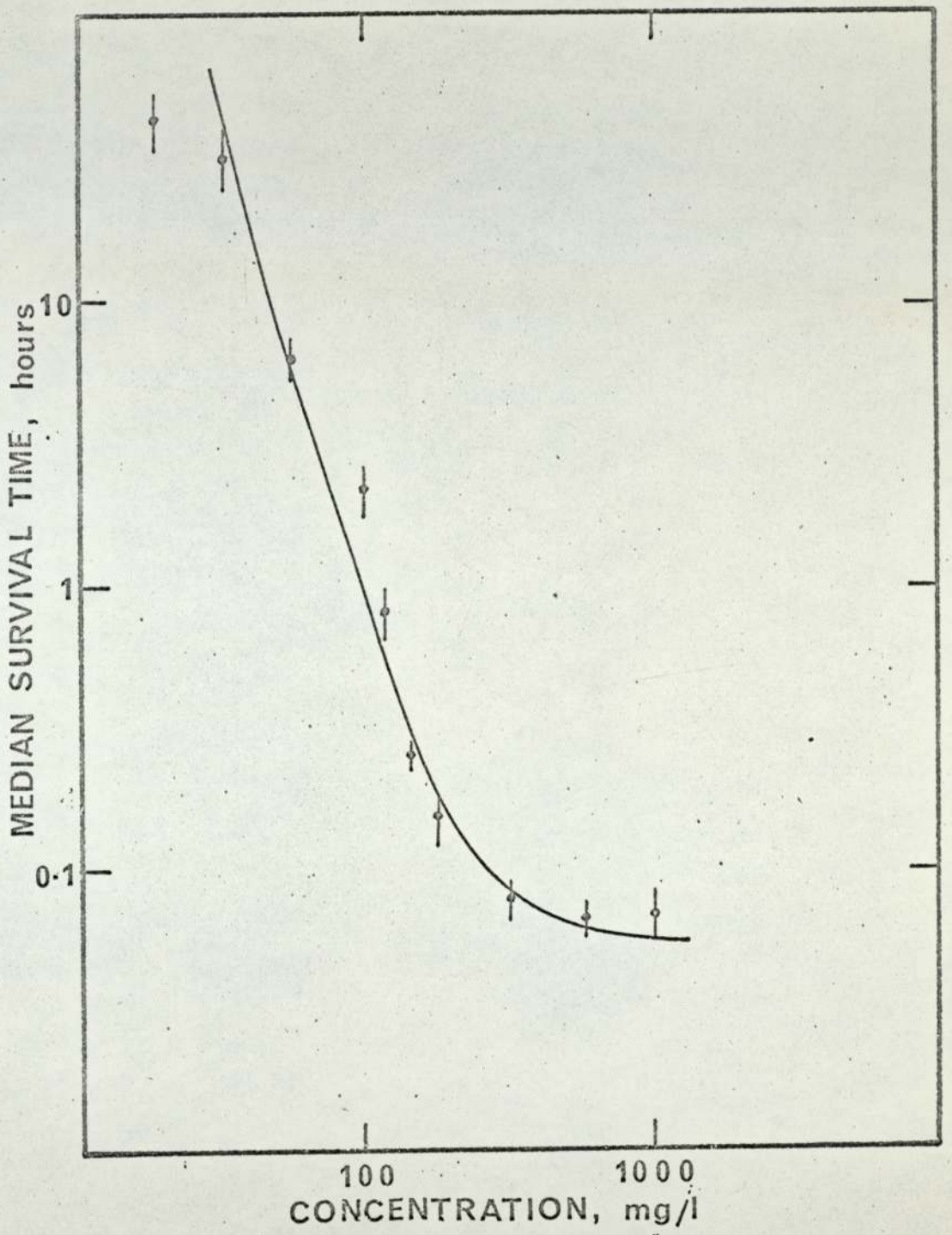


Fig. 4.3. Toxicity curve for Salmo trutta exposed to SLS.

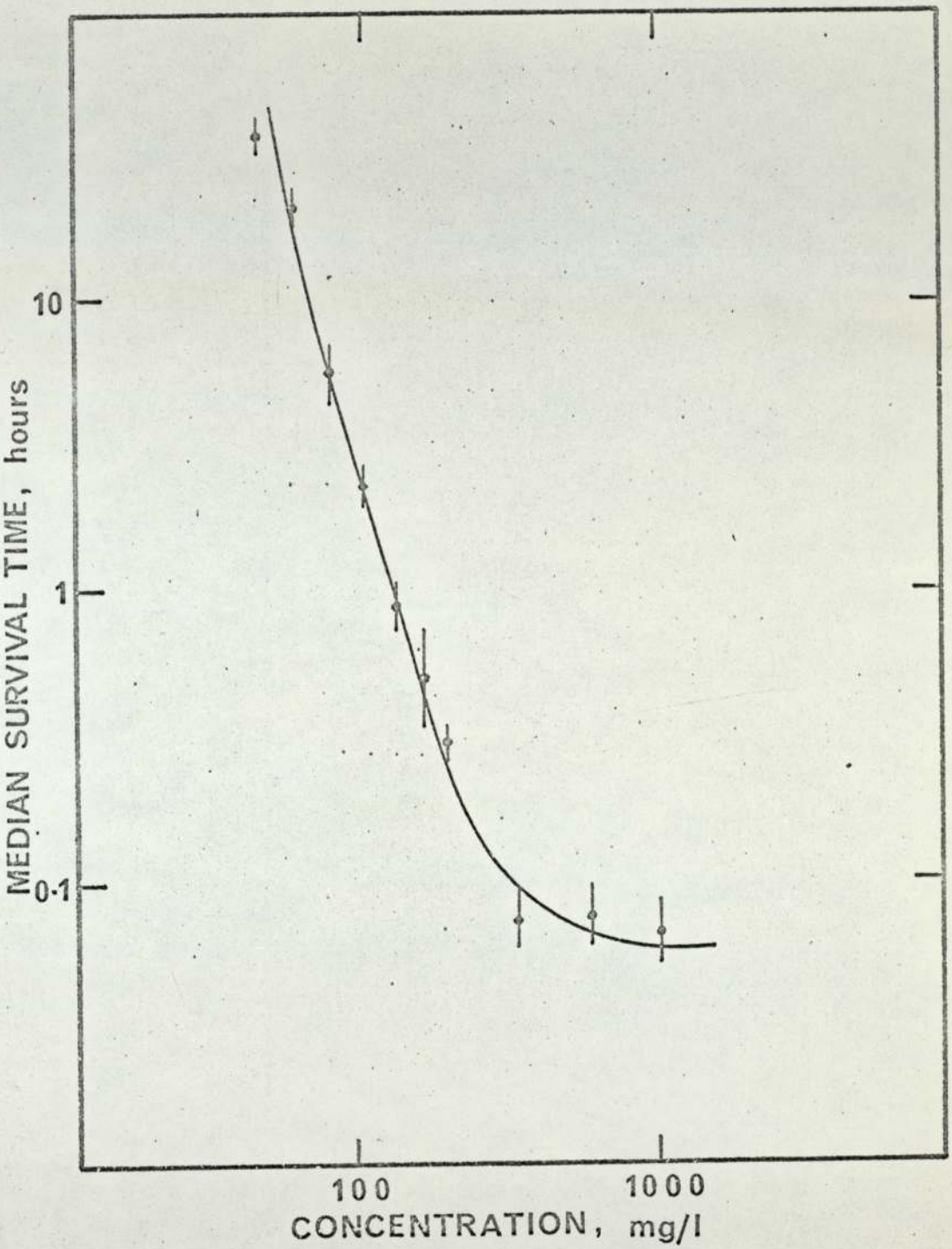


Fig. 4.4. Toxicity curve for Salmo gairdneri exposed to SLS.

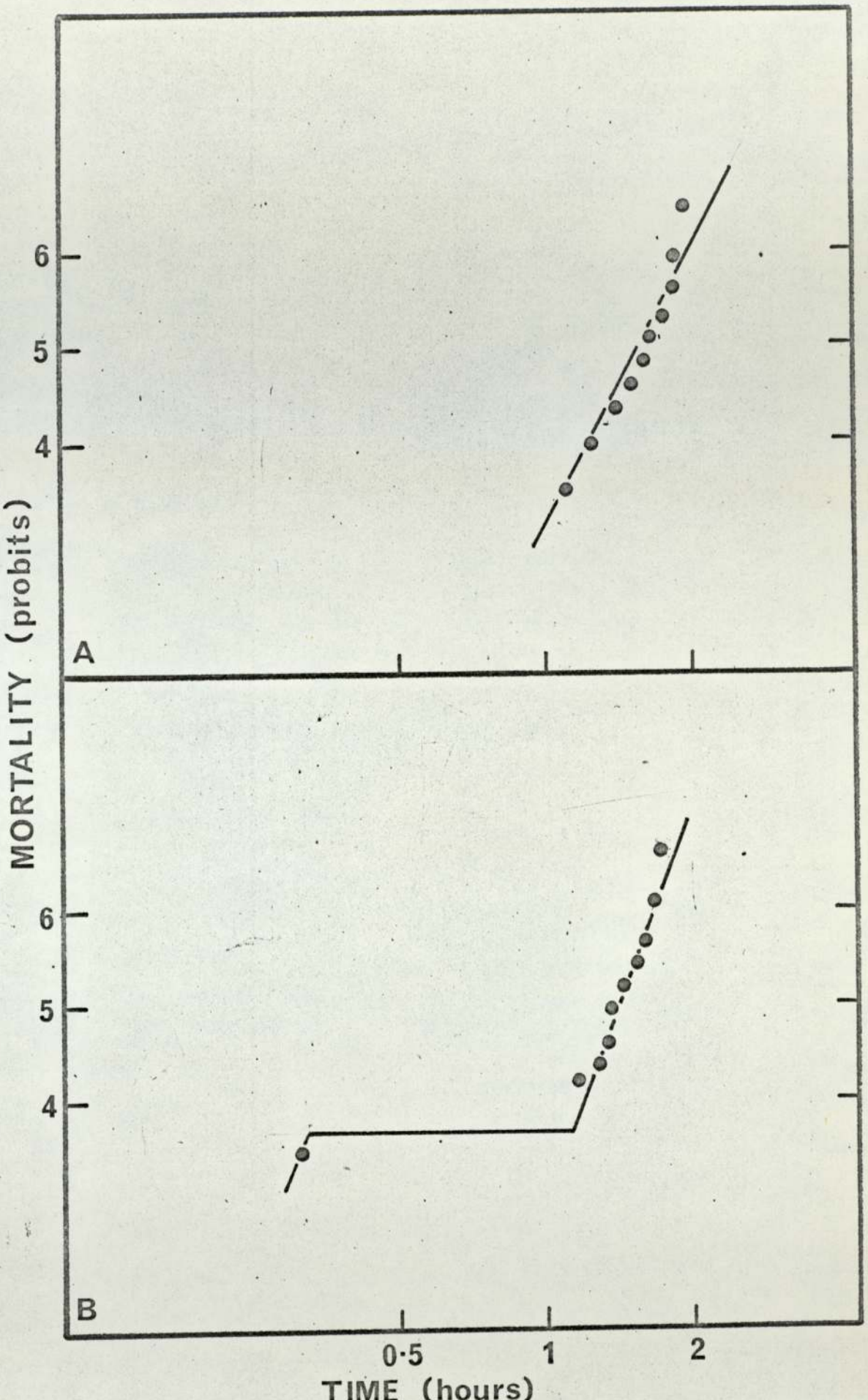


Fig. 4.5. Mortality curves for Salmo gairdneri exposed to 130 mg/l SLS.

CHAPTER 5. Toxic effects of sublethal concentrations of sodium lauryl sulphate and its residues on the gills of trout.

5.1 INTRODUCTION

Study of the pathological effects of sublethal concentrations of pollutants on fish tissues is an obvious technique for the assessment of the significance of pollutants at realistic concentrations. Gills are an important organ for study in this respect, since their fine structure is well known and they form the major area of contact between the fish and its environment. There have been only two previous electron microscopical studies of the effects of sublethal concentrations of pollutants on gills, those of Baker (1969) of copper, and Matthiessen & Brafield (1973) of zinc. The effects of sublethal concentrations of the anionic detergent sodium lauryl sulphate (SLS) on the gills of rainbow trout Salmo gairdneri, have therefore been studied, as an adjunct to the preceding work with higher concentrations and as a contribution to the assessment of the value of electron microscopical examination of the gills as an indicator of sublethal toxic effect.

The study includes a comparison of the effects of sublethal concentrations of whole detergent and the effects of similar concentrations of partially-degraded SLS, and has three objectives:

(a) To determine the extent to which the modes of toxic action observed at higher concentrations are operative at low concentrations.

(b) To determine whether there are any specifically long-term effects of detergents detectable by examination of the gills.

(c) To determine whether the concentrations tested are likely to be deleterious to the fish.

5.2 MATERIALS AND METHODS

Yearling Salmo gairdneri, mean fork length 12.6 cm (s = 1.9 cm), mean weight 25.6 g (s = 9.8 g) were obtained from a commercial hatchery and acclimated to Birmingham tap water (total hardness 25 mg/l as $CaCO_3$) at 15 $\pm$ 1° C for seven days before experiments began. Fish were fed

daily on pelleted food both before and during the experiments.

Fish were exposed in batches of eight to nominal concentrations of 32 mg/l and 18 mg/l SLS for seven days, and to a nominal concentration of 10 mg/l SLS for 15 days, in steel-framed glass aquaria containing 40 litres of test solution, gently aerated, at 15 $\pm$ 1° C. Each day 30 litres of solution was siphoned out and replaced with fresh solution.

Partially-degraded detergent was obtained by allowing aquaria containing 40 litres of a 50 mg/l solution of SLS to stand, vigorously aerated, at room temperature for 48 h, by which time the concentration of SLS as measured by the methylene blue method (see below) had fallen to about 20 mg/l. This solution was then transferred to a polythene container wherein a group of twelve fish were held for 28 days in 60 litres of test solution containing a nominal 10 mg/l of partially-degraded detergent. This tank was allowed to stand at ambient temperature (10 - 18° C). Each day 45 litres of solution were removed and replaced with fresh solution.

Detergent concentrations were monitored using the methylene blue method of Degens et al., (1953). Concentrations were determined daily for the first 7 days of the experiments and thereafter at least every 72 h, immediately before and after replenishment of the test solution.

At the end of each experiment, gill tissue was collected, prepared and examined by light and electron microscopes by the methods described in Chapter 2.

5.3 RESULTS

A. Detergent concentrations.

The detergent concentrations fluctuated considerably during the experiments, as shown in Table 5.1, and were generally rather lower than their nominal values.

B. Normal gill structure

Figs. 5.1 and 5.2 show light and electron micrographs of control S. gairdneri gill. The normal gill structure was described in Chapter 2. The principal features of interest here are:

(a) The epithelium is normally closely apposed to the underlying tissue, though some subepithelial spaces do occur and these occasionally contain lymphocytes.

(b) Chloride cells are plentiful in the basal regions of the lamellae and in the crypts between them, but normally do not occur to any large extent in the middle and distal regions of the lamellae.

C. Effects of SLS

The effects of sublethal concentrations of SLS are shown in Figs. 5.3, 5.4, 5.5 and 5.6. The outer layer of epithelial cells was extensively detached from the gill lamellae (Figs. 5.4 and 5.5). In addition, the epithelial cells were generally somewhat swollen, resulting in a large increase in the thickness of the water - blood pathway. Chloride cells were very common in the middle and distal regions of the lamellae (Fig. 5.5), as distinct from their confinement in control gill to the proximal lamellar regions and the interlamellar crypts. However, there did not appear to be any overall increase in the total number of chloride cells present.

The subepithelial spaces contained, in addition to an increased number of lymphocytes, considerable numbers of granulocytes (Fig. 5.4), which are never found in the subepithelial spaces of normal gill tissue. At the bases of the lamellae the filament epithelium showed slight oedema and leucocytic infiltration (Figs. 5.3, 5.6), as in a typical inflammatory response.

Gills of fish exposed to partially-degraded SLS for 28 days were similar to those of fish exposed to whole detergent, except that granulocytes did not occur in the subepithelial spaces (Fig. 5.7).

Neither in fish exposed to degraded detergent nor in those exposed to partially-degraded detergent was there any indication of cellular death occurring by the process of detergent-induced autolysis described in Chapters 2 and 3. There was no abnormal incidence of nuclear pyknosis, lysosome formation or cell sloughing, these features being very rarely observed as is typical of control gill.

Measurements of the total thickness of the water - blood pathway were made from electron micrographs and are given in Table 5.2. Between 70 and 100 measurements were made in each case, each measurement being made from the mid-point between adjacent pillar cells to the nearest point on the external epithelial surface. Obliquely-cut sections, as indicated by the appearance of the pillar cells, were not measured.

5.4 DISCUSSION

Epithelial swelling and lifting has been commonly reported as an effect of sublethal concentrations of pollutants on gills, both for detergents (Cairns & Scheier, 1962; Lemke & Mount, 1963; Brown et al., 1968) and for zinc (Brown et al., 1968; Matthiessen & Brafield, 1973), suspended solid particles (Herbert & Merkens, 1961) and the pesticide Endrin (Eller, 1971), but features possibly more specific in nature have also been found. Baker (1969) reported vacuolation in the pillar cells and epithelial cells in the gills of Pseudopleuronectes americanus exposed to sublethal levels of copper for about 4 weeks. Matthiessen & Brafield (1973) found cytoplasmic abnormalities in the gill epithelial cells of sticklebacks (Gasterosteus aculeatus L.) exposed to sublethal concentrations of zinc for 4 weeks, including the formation of membrane-bound vesicles and electron-dense accumulations of metabolites. Both Baker (1969) and Matthiessen & Brafield (1973) reported an increase in number and a redistribution of chloride cells so that they were found in the distal lamellar regions. In both cases the authors suggested that the chloride cells may be directly involved in the excretion of the toxic metal ions. In the present study, sublethal concentrations of SLS and

its residues were found to induce a redistribution of chloride cells, but there was no obvious increase in their numbers. Whether the chloride cells are involved in the metabolism of SLS, or whether SLS places an abnormal osmo-regulatory stress on the fish is not known.

No feature of the response of the gills to sublethal concentrations of SLS was detected which could be regarded as specific to SLS or any particular group of poisons. Although inflammation has not been reported in previous investigations of sublethal pollutant effects, the inflammatory response itself is inherently non-specific. However, it would be interesting to know the extent to which other poisons are able to induce inflammation at sublethal concentrations. The absence of granulocytes from the subepithelial spaces in gills of fish exposed to SLS residues may be related to a difference in toxic properties between whole and degraded detergent, but is more likely to be due to the fact that the detergent residue experiment lasted 28 days, while fish exposed to whole detergent were examined after 7 or 15 days. It is characteristic of subacute inflammation that granulocytes do not persist after 2 to 3 weeks (Anderson, 1966).

In experiments with 150-day old Salmo trutta (Chapter 3), concentrations of SLS down to 18 mg/l were found to induce autolysis in gill cells. This effect was not evident in yearling S. gairdneri exposed to similar concentrations of SLS, although at 100 mg/l SLS both types of fish showed similar gill responses (Chapters 2, 3). Possibly the differing ability to metabolise and detoxify fairly low concentrations of SLS is a function of species or age of fish.

The increase in thickness of the water - blood pathway may be expected to hinder respiratory exchange. The extent of this handicap may be estimated approximately by calculations of the type given below. According to their original authors, the results of such calculations are in reasonable agreement with experimentally determined values. It can therefore be shown whether the respiratory impediment caused by an increase

in thickness of the water - blood barrier is of a significant order of magnitude.

Suppose that the water - blood pathway is normally 1.5μ thick, and it increases as a result of pollutant action to 4.5μ. This is a reasonable supposition in the light of the results expressed in Table 5.2. The amount of oxygen diffusing across the gills is given by

$$Q = \frac{DA\Delta P}{L} \qquad (1)$$

where Q = amount of oxygen diffusing across the gills

A = area of the gills

ΔP = difference in oxygen partial pressure across the gills

L = thickness of the diffusion barrier

D = oxygen diffusion coefficient.

It must be stressed that L, the thickness of the diffusion barrier, is not the same as the thickness of the water - blood pathway. Oxygen must pass from the water across the blood - water pathway into the red blood corpuscles in the lamellar blood space. Since this space is just large enough to allow the passage of red blood cells, we may assume that the portion of the diffusion pathway within the blood space lies totally within a red blood cell. Since water flow through the gill sieve is laminar rather than turbulent (Hughes, 1966), there must also be a portion of the diffusion barrier lying in water. The maximum value of L will therefore be half the width of the interlamellar water space, plus the thickness of the water - blood pathway, plus half the width of the lamellar blood space. The mean value of L will be a quarter of the width of the interlamellar water space, plus the thickness of the water - blood pathway, plus a quarter of the width of the lamellar blood space.

The thickness of a lamella will be the width of the blood space plus twice the thickness of the water - blood pathway. The blood space is about 10μ in width, making a total lamellar thickness of 13μ for our

model fish. An adult trout has between 20 and 25 lamellae per millimetre of filament (Hughes, 1966; Steen, 1971), say 23. Assuming a lamella thickness of 13μ, the interlamellar space would be about 30μ wide. The mean value of L would therefore be

$$7.5\mu + 1.5\mu + 2.5\mu = 11.5\mu$$

If the thickness of the water - blood pathway increased by pollutant action to 4.5μ, lamellar thickness would increase to 19μ and the interlamellar water space would be correspondingly reduced to a little over 24μ. The mean value of L would therefore increase to

$$6\mu + 4.5\mu + 2.5\mu = 13\mu$$

The value for D, the diffusion coefficient, is a composite one, reflecting the relative proportions of L represented by water and tissue. Since an increase in the thickness of the water - blood pathway increases the proportion of L represented by tissue, the increase in the mean value of L will be accompanied by a change in the value of D. Values of D usually used for this purpose are those given by Krogh (1941), and are 0.000034 ml/cm^2/min for water and 0.000011 ml/cm^2/min for connective tissue (Hughes, 1966). For our normal fish 7.5μ of the mean value of L are in water and 4μ in tissue, giving an overall mean value for D of 0.000026 ml/cm^2/min. For a fish whose water - blood pathway has increased to 4.5μ, 7μ of L are in tissue and 6 in water, giving an overall mean value for D of 0.000021 ml/cm^2/min.

The combined effect of these changes in D and L is shown by Equation 1 to be a decrease in Q of approximately 34 per cent, i.e. a fish whose gill epithelium was affected by pollutant action to the extent assumed in this model would obtain only two-thirds the oxygen for a given respiratory effort that would be obtained by a normal fish under similar conditions. However, the calculations assume that the increase in the gill epithelial thickness is largely through tissue swelling rather than epithelial lifting; a large fluid-filled space probably approximates water rather than tissue in its diffusive capacity. It is also assumed

that tissue swelling does not appreciably alter the oxygen diffusion coefficient for the tissue. It is possible, however, that a swollen cell has a higher diffusion coefficient than a normal one, because as a general rule the diffusion coefficient of a tissue is given approximately by multiplying the diffusion coefficient for water by the percentage water content of the tissue (Steen, 1971). Therefore the estimate of a 34 per cent drop in oxygen uptake across the gills of an affected fish is best regarded as a maximal estimate.

Obviously any significant drop in the quantity of oxygen across the gills would stimulate the fish to take compensatory action, in the form of increasing the amount of water pumped over the gills. Since oxygen utilisation drops as ventilation volume increases, the extra amount of water required will be more than proportional to the drop in oxygen uptake enforced by the thickening of the water - blood pathway.

Increase in the thickness of the water - blood barrier would also, by decreasing the width of the interlamellar water spaces, alter the resistance of the gill sieve to the flow of water. The extent to which this might occur can be shown by using the modified Poiseuille equation originally used by Hughes (1966) to calculate the flow of water through the gills.

$$q = \frac{p_1 - p_2}{\eta} \qquad \frac{5d^3b}{24\,l} \qquad (2)$$

where q = the flow of water through each pore in cubic centimetres per second

$p_1 - p_2$ = the pressures, in dynes per square centimetre, on either side of the gill sieve.

η = the viscosity of water, in poises.

d = width of the interlamellar water space (cm).

l = length of a secondary lamella (cm).

b = height of the interlamellar water space (cm).

Using the values given by Hughes (1966) for a trout weighing 175 g,

$$q = \frac{500}{0.01} \times \frac{5\,(0.0023)^3 \times 0.04}{24 \times 0.07}$$

$$= 0.72 \times 10^{-4} \text{ cc/pore/sec.}$$

If the water - blood pathway increases in thickness by 3μ, the interlamellar water space decreases in width by 6μ, so that d becomes 0.0017 cm. In this case it is immaterial whether the increase in thickness of the barrier is caused either by tissue swelling or epithelial lifting. The result of the change specified above is a decrease in q to 0.30×10^{-4} cc/pore/sec, less than half the normal flow.

Therefore in order to pass a given amount of water over its gills, the fish would have to increase the buccal pressure to about twice its normal value. Energy is also required to pump water into the mouth and out via the opercula, and these energy values would not be affected. The increase in gill resistance would demand an increase in (p_1-p_2) from about 0.5 cm water to about 1.0 cm water. According to the values given by Alexander (1967), this would represent an increase of about 50 per cent in the total energy cost of water pumping.

So a fish whose gills were affected by pollutants such that the gill epithelium was swollen or abnormally detached would be forced to pump more water at increased energy cost over its gills. An active fish in well-oxygenated water requires about 15 per cent of its total energy expenditure simply to pump water over its gills (Alexander, 1967). The above calculations suggest that sublethal pollutant effects on gills may appreciably increase the energy cost of respiration. Clearly a respiratory handicap of this sort would have several implications. The tolerance of the fish to low dissolved oxygen would decrease, its maximum sustainable level of activity would decrease, ~~its maximum sustainable level of activity would decrease,~~ and less energy would be available for other metabolic functions. These effects are potentially

of profound ecological significance. It is possible that the effects of sublethal levels of pollutants on fish growth and reproduction may be due in large measure to the increased energy cost of respiration, quite apart from any specific toxic effects.

TABLE 5.1. Concentrations of sodium lauryl sulphate in the test tanks, as measured by the methylene blue method.

Nominal concentration	Maximum diurnal variation
32 mg/l SLS	30 mg/l - 10 mg/l
18 mg/l SLS	15 mg/l - 5 mg/l
10 mg/l SLS	10 mg/l - 4 mg/l
10 mg/l SLS residue	15 mg/l - 4 mg/l

TABLE 5.2. Thickness of the water - blood pathway in gills of *Salmo gairdneri* exposed to sublethal concentrations of SLS and SLS residues. Mean values obtained from between 70 and 100 measurements in each case.

	Thickness of pathway	
	Mean (microns)	Range (microns)
Control	1.1	0.5 - 2.6
32 mg/l	3.5	0.9 - 7.1
18 mg/l	2.8	0.9 - 6.8
10 mg/l	3.1	1.4 - 6.5
SLS residues	4.3	1.8 - 7.0

Fig. 5.1. Light micrograph of control gill. A small degree of epithelial detachment is normally found in control tissue (x 350).

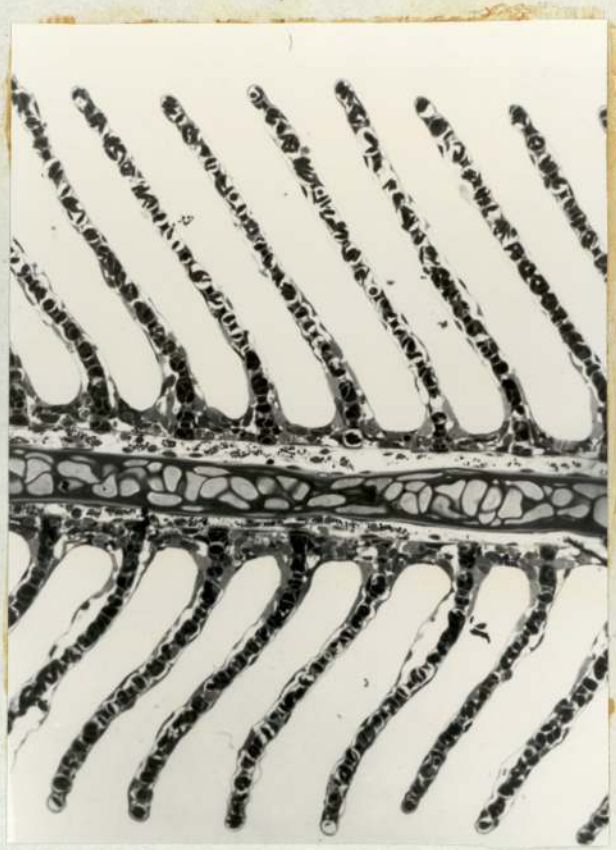
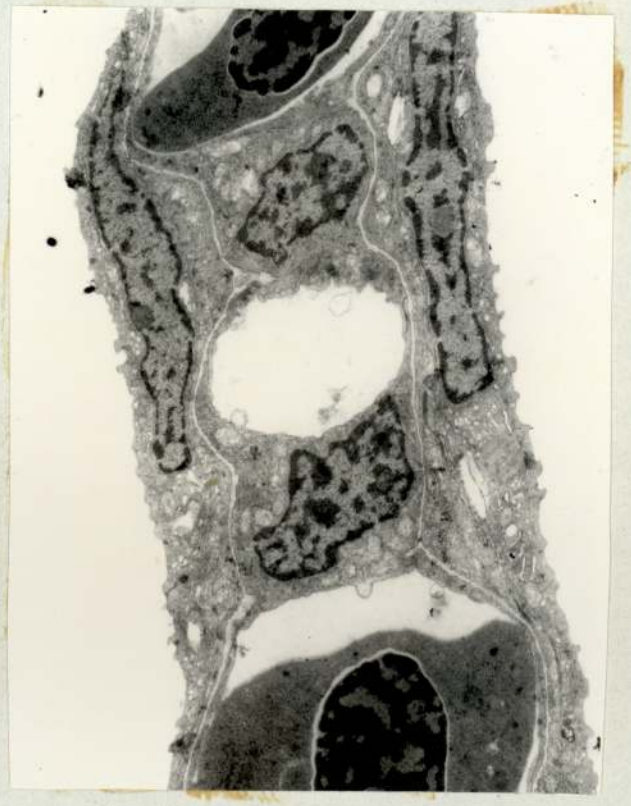


Fig. 5.2. Electro micrograph of control gill lamella (x 7500).



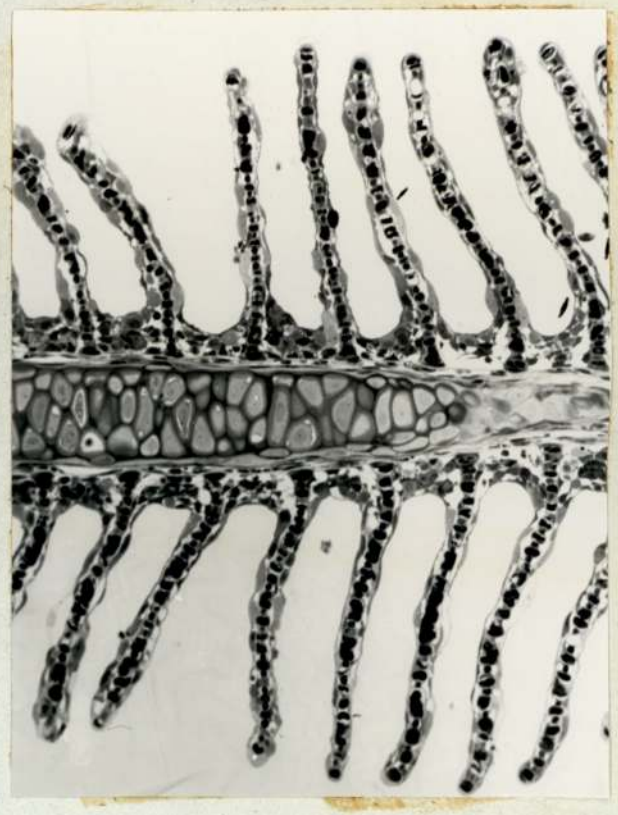


Fig. 5.3. Light micrograph of gill from fish exposed to 32 mg/l SLS for seven days. Note marked thickening of lamellar epithelium and oedema of filament epithelium. (x 300).

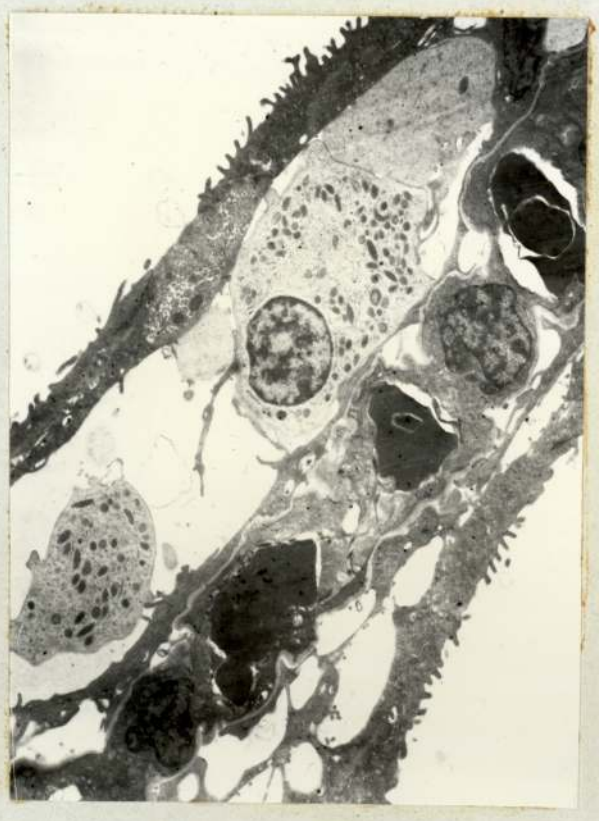


Fig. 5.4. Granulocytic invasion of the sub-epithelial spaces in a lamella from a fish exposed to 18 mg/l SLS for seven days. (x 4800).

Fig. 5.5. The redistribution of chloride cells into the lamellar mid - and distal regions contributes to the increase in thickness of the water - blood pathway. 18 mg/l SLS, seven days. (x 7500).

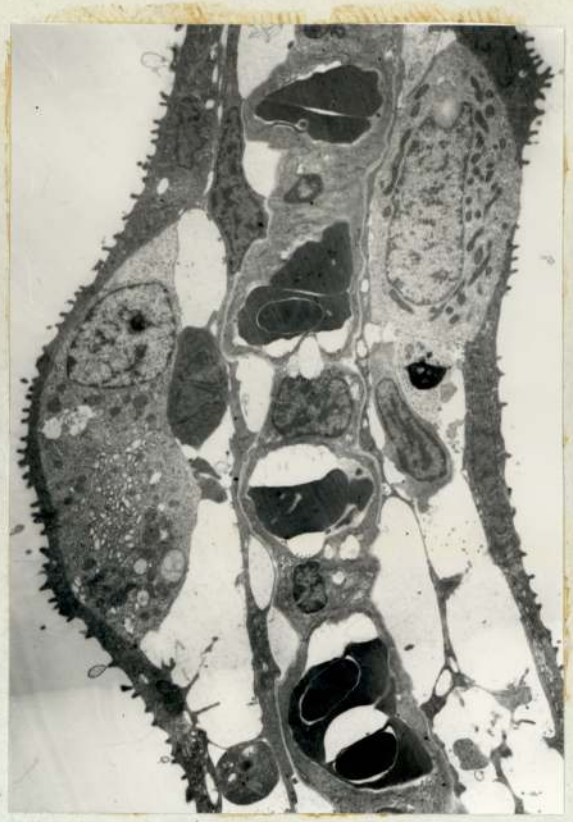
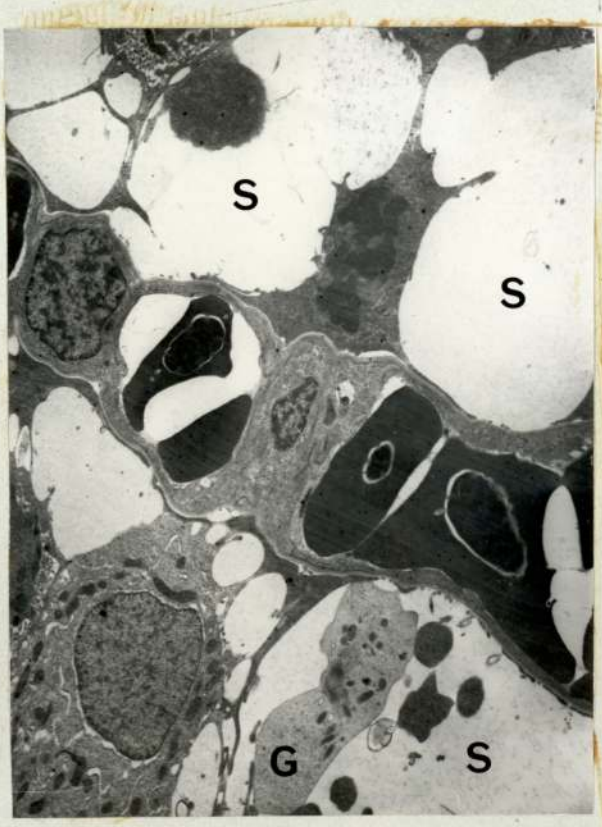


Fig. 5.6. Lamellar proximal region in the gill of a fish exposed to 18 mg/l SLS for seven days. Note large subepithelial spaces (S) and granulocyte (G). (x 5100).



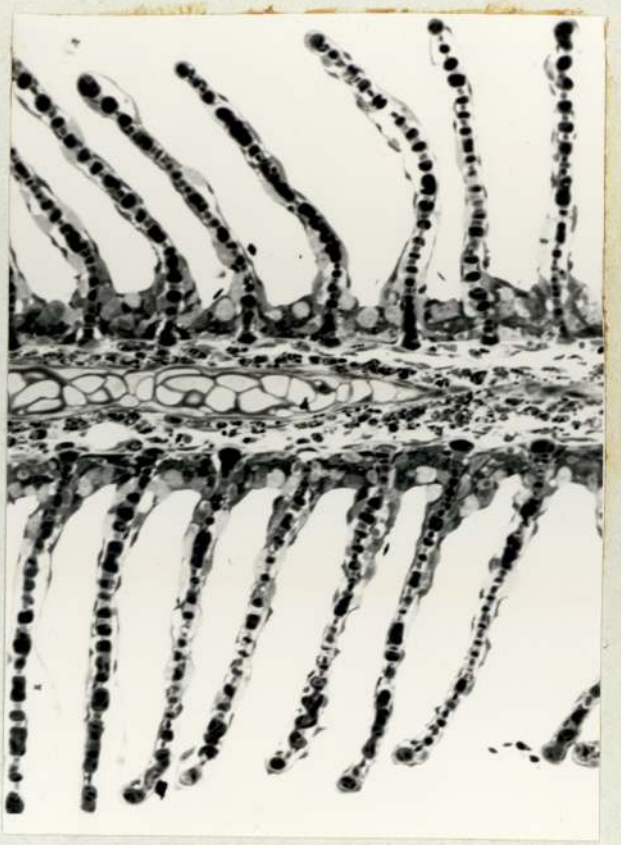


Fig. 5.7. Light micrograph of gill from fish exposed to SLS residues for 28 days. Note lamellar disarray and epithelial thickening. (x 300).

CHAPTER 6. General discussion.

The methodology and literature relating to the toxicity of pollutants to fish have developed rapidly in recent years, as the recent series of reviews by Sprague (1969, 1970, 1971) indicates. Major areas of interest, which are all inter-related, are toxicity testing and the interpretation of toxicity test results; the toxic effects of pollutants and the nature of their toxic actions; the responses of the fish to pollution and the nature of these responses; and the physiology of fish under toxic stress and its relation to normal physiology, especially in so far as information may be gained regarding normal physiological mechanisms. This thesis describes a series of investigations into the effects of a range of concentrations of a toxic substance on a vital organ in fish - the gills - and the implications of its results will be discussed in relation to the areas of interest outlined above. Those aspects of the investigation which may be of wider biological interest will also be indicated.

Study of the effects of the anionic detergent, sodium lauryl sulphate, on trout gills has produced evidence of three separate modes of toxic action. At sublethal levels (Chapter 5), the effect of the detergent was to increase the thickness of the water - blood pathway by causing epithelial swelling and an increased degree of lamellar epithelial lifting. Sub-acute inflammation of the gill tissue also occurred. These effects appeared to be non-specific, in as much as sublethal concentrations of other pollutants produce similar results. At higher detergent concentrations (Chapter 2), severe alterations in normal gill structure were effected. The immediate cause of this seemed to be a substantial degree of mortality among gill epithelial cells. Electron microscopical evidence indicated that cell death was due to an autolytic process initiated by detergent action (Chapter 3), and at these concentrations death of the fish eventually occurred. At very high concentrations, in excess of 120 mg/l (Chapter 3), rapid and severe disruption

of the gill tissue occurred. This process involved lysis of cells by the direct action of the detergent on the cellular constituents, and was associated with haemolysis in the circulating blood. Again, death of the fish resulted.

Detergent action on isolated cells such as bacteria and erythrocytes *in vitro* has long been thought to involve one or other of these two processes (Hugo, 1967). The existence of these mechanisms has been inferred from biochemical and other evidence, and does not appear to have been demonstrated previously by electron microscopy. This demonstration may be of some interest to biologists working at a more fundamental level. It also illustrates the potential value of drawing on the vast biomedical literature relating to the metabolism and effects of toxic substances in mammalian and prokaryotic systems. A recent example of this approach of more immediate value is that of Baker (1969), who found that the pathological effects of sublethal concentrations of copper in the flounder *Pseudopleuronectes americanus* were similar to a well-known syndrome of copper poisoning in mammals.

Histological techniques are widely used in assessing effects and the nature of their toxic action. Electron microscopy has been less extensively employed in fish work, there being only three previous studies involving its use (Baker, 1969; Skidmore & Tovell, 1972; Matthiessen & Brafield, 1973). As a review of the literature relating to pollutant action on gills (Chapters 2, 5) illustrates, conventional histological techniques have so far proved disappointing in that toxicant-specific effects have rarely been detected. However, more refined histological methods combined with a knowledge of toxicant-induced syndrome in other vertebrates, and the investigation of a suitably wide range of tissues may lead to the better definition of criteria of toxic effect in fish. The wider use of electron microscopy certainly seems desirable. Differences in toxic effect at the cellular level between SLS and zinc sulphate were readily observed (Chapter 2). A difficulty pointed out by

Sprague (1971) is that very little is known of the normal histology and ultrastructure of fish tissues, so that it is difficult to determine the significance of any changes which may be detected. This is likely to be especially important in relation to long-term, sublethal effects, and indicates the value of gills as an organ for study, since their normal structure and function are well known. Deviations from normal as a result of long-term exposure to SLS and its residues were easily detected (Chapter 5), and are to some extent amenable to quantitative evaluation. Although no quantitative relation between exposure and response was determined, it was possible to calculate that a respiratory handicap imposed on the fish by pollutant action could reasonably be expected to be of a significant order of magnitude.

The pathological effects of a poison in part represent the response of the fish to the action of the poison, and it is worthwhile to consider toxic effects from this point of view. At sublethal concentrations of SLS, the responses in the gill tissue were subacute inflammation, epithelial swelling and lifting, and redistribution of chloride cells (Chapter 5). Inflammation is generally accepted as a non-specific response to any chemical or physical stress, and in this instance is conceivably involved in detoxification. Insufficient is known of chloride cell function to know whether their redistribution indicates a role in metabolising the poison, or even whether it is evidence of an osmoregulatory stress. Epithelial swelling and lifting may serve to protect the fish by hindering pollutant uptake, although at the cost of respiratory efficiency. However, it is not known whether the swelling and lifting is under any form of central control, whether it is locally effected by pollutant action, or whether cellular metabolism is impaired, either as a cause or an effect of the swelling and lifting.

At lethal concentrations of SLS the nature of the response is easier to interpret. At concentrations up to about 120 mg/l, an acute inflammatory response occurs (Chapter 2), and as such is unremarkable.

The pattern of gill damage is explainable as a response to the loss of cell function at an important interface with the external environment. The loss of cells occurs at a rate which is sufficiently slow to allow some compensation. Dead and dying cells are sloughed off and replaced with functioning cells in order to preserve the integrity of the barrier between the internal and external environments. However, since cell death occurs more rapidly than the formation of new cells, the total area of the gill surface has to be reduced, and this can only be achieved by the extensive detachment of the lamellar epithelium. As indicated in Chapter 2, this raises some interesting physiological questions. At concentrations above about 120 mg/l, cell death occurs by a more rapid process and there is no possibility of any compensatory action. Therefore the gills retain their overall gross form even though cellular structure and function are totally destroyed.

In Chapter 4, it was shown that the data obtained in a conventional toxicity test could demonstrate the existence of the two separate modes of lethal toxic action indicated by the gill pathology. In general, it is unreasonable to assume that a poison has only one mode of action. Lethal concentrations of a poison may act within minutes or over several days. Sublethal concentrations may produce a range of effects such as behavioural changes, loss of fertility or serious debilitation, reversibly or irreversibly. The nature of the toxic action is probably not the same in all cases. Toxicity testing still claims a major part of the research effort of fish toxicologists, and although toxicity curves are frequently published, detailed consideration of probit lines seems rarely to be given. The findings of Chapter 4, that a changed mode of action may be indicated in the probit lines but not in the shape of the toxicity curve, suggest that a great deal of information about unusual toxicity relationships could be obtained if toxicity data were more fully scrutinised.

At this point it is pertinent to consider precisely what is meant by the term "mode of toxic action". Pollution toxicologists tend to use the term somewhat loosely, often to mean merely the description of the symptoms of toxic action. For example, to say that a poison acts by damaging the gills and preventing respiratory exchange merely begs the question of how the poison damages the gills. Many of the techniques and terms of fish toxicology are adapted from pharmacological practice. To a pharmacologist, the term "mode of action" refers to the biochemical lesion produced by the poison at the molecular level. Any consequence of the poison's presence other than at the molecular level is a toxic effect. The meaning of the term which is implicit in the foregoing discussion is that the mode of action of a poison on fish is the sequence of cause and effect at succeeding levels of biological integration. Such a definition has regard to the major objectives of studying the action of toxic substances on fish, which are the elucidation of physiological mechanisms and the application of toxicity data to situations of pollution in the field. It follows that any study of toxic effect, whether biochemical, histological, physiological, behavioural or ecological, is the study of one or more steps in a much longer sequence of events. This sequence includes the entry of the poison into the fish, its biochemical and physiological effects on the individual fish and, ultimately, their ecological implications.

Those steps in the sequence which most influence and are most influenced by the environmental and biotic factors which are normally limiting to the fish are likely to be the most crucial elements in the mode of toxic action from the point of view of water pollution. Thus the actual or potential significance of a given level of pollution can be assessed. This is the rationale behind Sprague's (1971) statement that "Understanding physiological action of a toxicant is the key to predicting important sublethal effects."

There have been no studies of the effects of detergents on physiological mechanisms in fish, although some such studies have been carried out for other poisons. Lloyd & Orr (1969) studied the effect of ammonia on the urine flow rate of trout. They found that ammonia increased the water permeability of the fish. This effect can be regarded as being a crucial step in the mode of action of ammonia, since it allowed the authors to conclude that any factor which affects water balance will also affect ammonia toxicity. This work also explained some features of ammonia toxicity determined in earlier experiments, such as the effect of salinity and handling and the non-contribution of low levels of ammonia to the toxicity of mixtures of poisons. Skidmore (1970) measured respiratory parameters and the ionic composition of trout exposed to zinc at a concentration sufficient to cause death within a few hours. Gill damage similar to that described in Chapter 2 was caused (Skidmore & Tovell, 1972). Death was due to asphyxia rather than osmoregulatory imbalance. This finding lends support to the interpretation of the nature of the gill response as an attempt to maintain the internal osmotic environment in the face of a progressive loss of cell function in the gill epithelium (Chapter 2). Packer & Dunson (1972) demonstrated that trout killed by exposure to low pH suffered a heavy sodium depletion, and that survival time was predictable from the rate of sodium loss. An isotonic medium prolonged survival. Oxygen consumption decreased progressively for unknown reasons - possibly gill damage or respiratory acidosis. In this case the relative contributions of respiratory and osmotic stress were not determined. Studies such as these in relation to detergent action would be highly desirable, to investigate the physiological action of the detergent and the nature of the homeostatic failures which result in death. It would also be interesting to know whether detergents enter fish and by what routes, and the extent of their internal toxic action.

It is hoped that the investigations described in this thesis will

be of interest in several areas of biology and applied biology. In the absence of physiological investigations into detergent action, Chapters 2 and 3 may be of limited interest to the pollution biologist, although they have raised some questions concerning fish physiology in general. Interest in all aspects of fish pathology is currently widespread, and the action of toxic substances must be included in this field of research. The interaction of detergents and living matter has been an active area of research for many years, particularly in the study of membrane and protein structure and cell physiology. Detergents are widely used as bacteriocides and are also in general domestic use in high concentrations, so the study of their toxic action is a matter of some public health importance. Chapters 4 and 5 are of greater interest to the pollution biologist, Chapter 4 in relation to the interpretation of toxicity tests and Chapter 5 in relation to the possible effects of sublethal concentrations of pollutants on the efficiency of respiration. It is suggested that further studies of detergent action on fish would most profitably involve physiological investigation.

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